Identification of SeqA interaction partners by searching for multicopy suppressor genes

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Karoleen Kamlow

SUMMARY

The *Escherichia coli* (*E. coli*) DNA replication is initiated at the origin of replication, *ori*C. The newly replicated DNA are hemimethylated, where the parental DNA strand is methylated and the new DNA strand is unmethylated. The new DNA remains hemimethylated for up to 1/3 of the cell cycle by a process known as sequestration. The SeqA protein has been identified as an important factor in sequestration, by binding to the hemimethylated origins and preventing reinitiation of replication. The SeqA protein is therefore known as a negative modulator of initiation of replication in *E.coli*.

It has been assumed that SeqA does not operate alone, and perhaps there are proteins that interact with it, thus promoting SeqA's functions. Biochemical experiments have identified three membrane proteins that were present in wild type *E. coli* cells, but not in Δ*seqA* mutant cells. These proteins might be potential interacting partners for the SeqA protein.

Here we have used a genetic screen as an independent method to attempt to identify SeqA interacting partners. The genetic screen involved making an *E.coli* genomic library and transforming it into a temperature-sensitive strain with double mutation, *seqA4*Δ*recA*.

The *seqA4*Δ*recA* strain was viable at 42°C, but not at 30°C. DNA fragments from the genomic library were cloned into a multicopy plasmid and transformants were then selected at 30°C. The cells that would form colonies at 30°C would therefore contain a multicopy suppressor gene coding for a SeqA interacting protein. We found that the *seqA4*Δ*recA* strain accumulated suppressors when it was transformed with an empty plasmid (background frequency). This made the screen extremely challenging. The suppression-mediated growth was observed after 2 days of incubation.

Furthermore, some preliminary results were found. During investigation of the background frequency of suppression, an interesting phenotype was observed where the vector (pUN121) was reduced in size. It is suggested that this suppressor mutation may affect replication fork repair or degradation.

ABBREVIATIONS

°C	Degrees Celsius
bp	Base pairs
c.f.u.	Colony forming units
dH_2O	Sterile water
dsDNA/ssDNA	Double/single stranded DNA
DNA	Deoxyribonucleic acid
et al.	With others (Latin alibi)
h	Hour/hours
In vitro	Process acting in a reaction tube
In vivo	Process acting in the cell
Kb	Kilobases
kDa	Kilodalton
(l)	Liquid
mM	Millimolar
OD	Optical density
µg/µl	Microgram/microliter
mg/ ml	Milligram/milliliter
rpm	Revolutions per minute
ssDNA	Single stranded DNA

CONTENTS

1 INTRODUCTION

1.1 *Escherichia coli*

Escherichia coli (E. coli) is a gram negative, rod-shaped bacterium and is practically the most studied microorganism (Pallen and Nelson 2007). Due to its rapid growth rate and simply nutritional requirements, *E. coli* has become biology's primary model organism. Over the past years, studies on *E. coli* has given us a better understanding about important mechanisms involved in DNA replication and regulation.

1.2 The *E. coli* cell cycle

The cell cycle of *E. coli* involves growing and dividing the cell into two daughter cells, where each of the daughter cells contains the same amount of DNA. The rate of bacterial growth and division depends on environmental conditions, such as growth medium and temperature (Donachie 1993).

E. coli contains one circular chromosome, which replicates before cell division. The cell mass of *E. coli* increases during the cell cycle, and once it reaches a specific cell mass, DNA replication initiates (Donachie 1993). Replication is initiated at *ori*C (origin of replication; see Section 1.3.1), and DNA is synthesized at a constant rate, bidirectionally around the *E. coli* chromosome (Figure 1). The replication ends in the terminus region where replication forks meet. The two sister chromosomes separate before cell division where the cytoplasm separates to form two compartments resulting in two daughter cells each containing one copy of the chromosome (Donachie 1993).

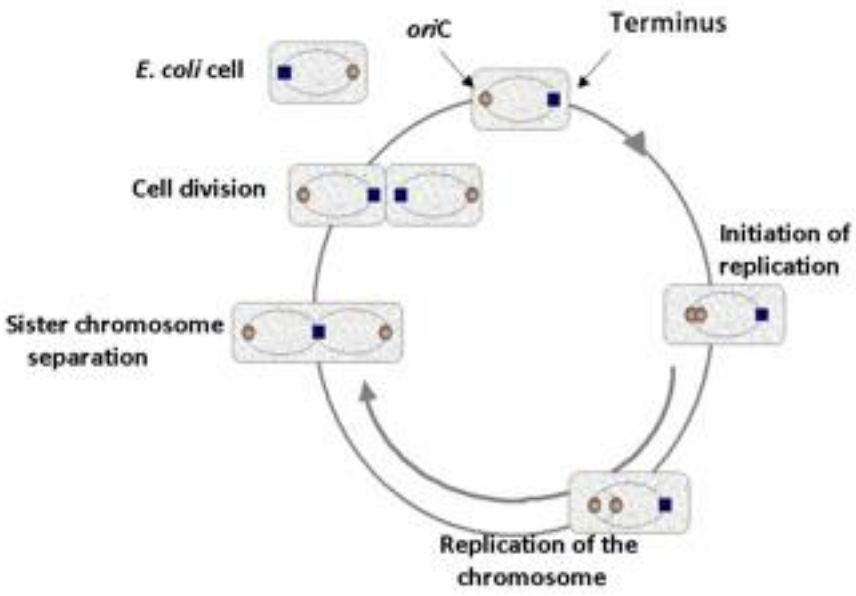

Figure 1: *E. coli* cell cycle during slow growth. The circles represent the circular chromosome in *E. coli*. The dots represent origin of replication, *ori*C, and the squares represent the terminal region. During cell cycle the chromosome is replicated and separated, and the cell is divided into two daughter cells, each containing one circular chromosome. This figure is drawn by Ingvild Flåtten.

The growth rate of *E. coli* varies between slow-, moderate-, and rapid. Cells with slow growth rate require longer than 60 minutes for doubling the amount of the cell mass (doubling time). However, cells with a moderate growth rate double the cell mass in 40 minutes and cells with a rapid growth rate double the cell mass in 20 minutes (Baker and Kornberg 1992).

1.3 DNA replication

DNA replication is a semiconservative process that involves the formation of complementary DNA strands as a result. The DNA replication proceeds from the origin site, where replication forks move bidirectionally until they reach the terminus point at the end of the DNA replication (Baker and Kornberg 1992). Figure 2 shows a scheme of a replication fork during DNA replication, involving different proteins controlling different events.

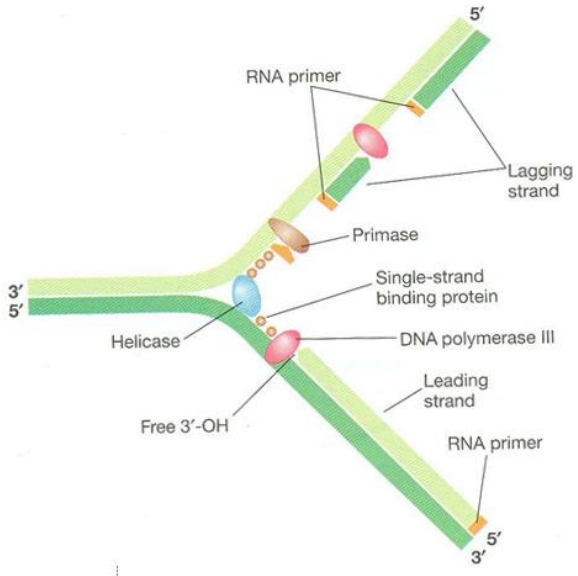

Figure 2: Events at the DNA replication fork. DNA-helicase unwinds the double helix of DNA resulting in exposed single stranded region. DNA-polymerase synthesizes the DNA in 5'→ 3'direction resulting in leading and lagging strands. This figure is from (Madigan, et al. 2008).

1.3.1 Origin of replication, *ori*C

Escherichia coli contains one circular chromosome that must be replicated once per cell cycle (Boye, et al. 2000). The replication occurs at a unique chromosomal replication origin referred to as *ori*C. The minimal *ori*C region that is required for initiation of replication is found to consist of 245 base pairs (Zyskind and Smith 1986).

Figure 3 illustrates the *E. coli ori*C, where (■) represents the regions with GATC sequences. Minimal *ori*C contains 11 GATC sites in separate locations within *ori*C. The GATC-sites are recognized by DNA methyltransferase (Dam methylase) and methylated at the N^6 position of adenine (Marinus 1996).

The left end of *ori*C is the DNA unwinding region and contains three AT rich 13-mer sequences. The initiator protein, DnaA, interacts with the AT rich region with low affinity (Baker and Kornberg 1992; Leonard and Grimwade 2005). Next to the AT rich region, there are five main high affinity binding sites for the initiator protein, DnaA, which are termed as DnaA-boxes R1-R4 and M (Fuller, et al. 1984; Schaper and Messer 1995) (Figure 3).

The IHF binding site lies between the DnaA boxes R1 and M. The IHF protein is known to induce bending of DNA and stimulates the opening reaction of the DNA double helix (Skarstad, et al. 1990; Torheim and Skarstad 1999), see Section 1.3.3.

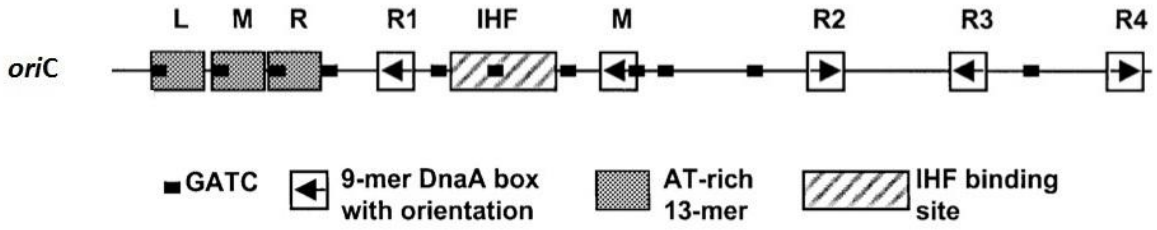

Figure 3: The *E. coli ori*C region. *ori*C consists of 11 GATC-sequences (■), the AT rich 13-mer region (L,M and R), and DnaA boxes R1-R4 and M. The IHF-binding site is between R1 and M (DnaA boxes). The figure is modified from (Torheim and Skarstad 1999).

1.3.2 The initiator protein, DnaA

The DnaA protein is a monomer of about 50 kDa, encoded by the *dnaA* gene. DnaA belongs to a large family of proteins with common sequence motifs, the AAA+ family of ATPases. This protein family has functions essential for the initiation of DNA replication (Messer 2002).

The initiator protein, DnaA, plays an important role in DNA replication .DnaA binds to the AT-rich region in *ori*C (Figure 3), unwinds the DNA duplex and recruits two DnaB helicase molecules to each of the separated DNA strands forming the initiation complex (Fang, et al. 1999; Carr and Kaguni 2001).

The DnaA protein has two conformations, active and inactive. The active form is DnaA bound to ATP (ATP-DnaA), whereas the inactive form is DnaA bound to ADP (ADP-DnaA).

1.3.3 Molecular mechanism of initiation of DNA replication

The DNA replication involves several stages with different proteins having specific activities in the replication reaction. The following steps are involved in replication initiation (Figure 4):

1. Formation of the initial complex: In this first step the initiator protein, DnaA, recognizes and binds to the five 9-mer DnaA-boxes in *ori*C. Both ATP-DnaA and ADP-DnaA bind to DnaA-boxes with the same affinity, however, only ATP-DnaA binds and unwinds the DNA duplex at the AT rich region (Messer 2002).

2. Unwinding the DNA duplex: Further binding of ATP-DnaA to the AT-rich region leads to unwinding of the DNA duplex. The HU or IHF proteins are required for this reaction to proceed, along with high level of ATP and presence of negative supercoiling (Messer 2002).

3. Loading of DnaB helicase: After unwinding and separation of the strands, two DnaB-helicase molecules are loaded to each of the single strands. DnaB helicase exists in a hexameric form, bound to six molecules of DnaC, the helicase loader (Funnell, et al. 1987; Fang, et al. 1999; Watt, et al. 2007).

4. Translocation of DnaB helicase and primase loading: Once the DnaB helicase molecules are loaded on the unwounded single strands, they move along the strands in 5'-3' direction creating a bubble of about 65 nucleotides (Fang, et al. 1999). Afterwards, primase enters the complex and interacts with DnaB helicase to form the priming complex by synthesizing primers on each strand (Messer, et al. 1988; Lu, et al. 1996; Fang, et al. 1999).

5. Replisome complex: In this final stage, a replisome complex is formed by loading two DNA polymerase III molecules onto each primed template in order to copy leading and lagging strands at the replication forks. The replisome complex consists of the polymerase III core (contains DNA polymerase) and the β-clamp (confers processivity to the polymerase by holding the polymerase III core onto DNA). The leading strand is synthesized continuously by DNA polymerase III, by adding new nucleotides to the free 3'OH at the replication fork (Baker and Kornberg 1992) (Figure 2). However, the lagging strand is synthesized discontinuously in short segments called Okazaki fragments, which later are joined together by ligase and forming a continuous DNA strand (Baker and Kornberg 1992).

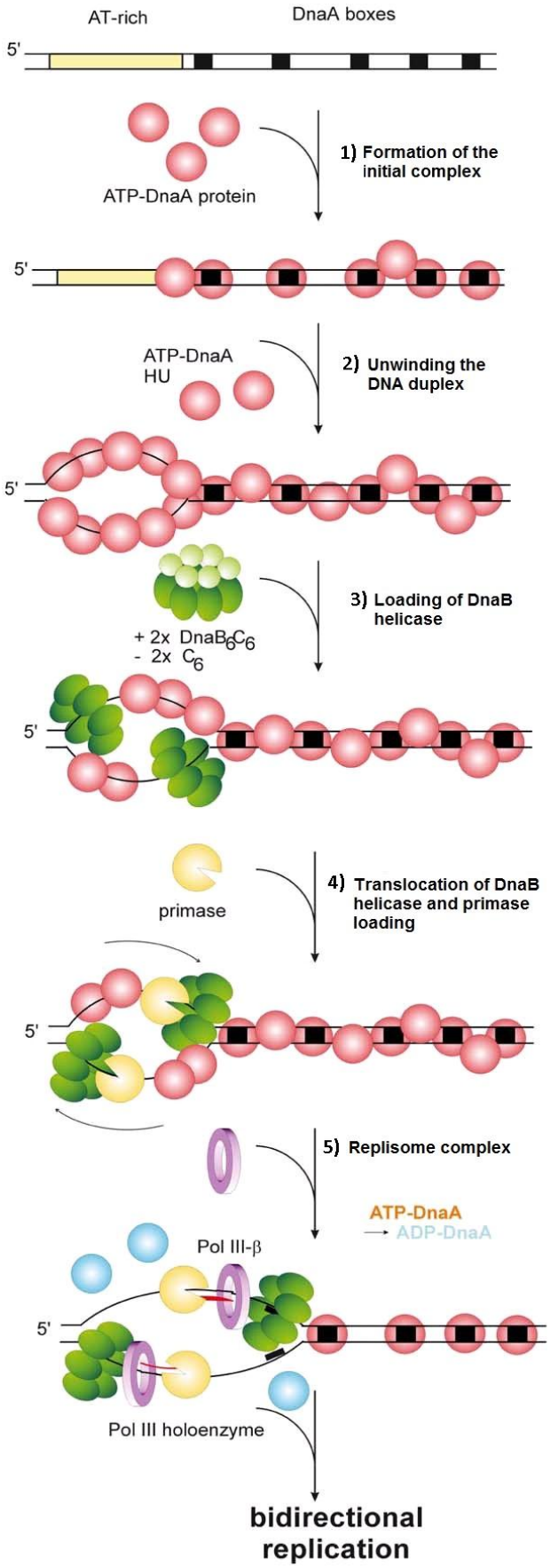

Figure 4: Initiation of replication in *E. coli*. Initiation of replication involves 5 steps where the formation of the open complex is the first step, followed by recruiting and binding of DnaB helicase and the rest of the replication machinery to form two new DNA strands. The figure is modified from (Messer 2002).

1.3.4 Regulatory mechanisms of initiation of DNA replication

Prior to cell division, the DNA must be replicated once per cell cycle (Boye, et al. 2000). Cells have mechanisms to prevent reinitiation of origins that have already been initiated. In *E. coli*, there are three mechanisms that regulate this event: sequestration, inactivation of DnaA and titration of DnaA.

1.3.4.1 Sequestration

Sequestration is a process that prevents reinitiation of newly replicated origins (Campbell and Kleckner 1990). There are 11 GATC sequences spread along the replication origin of *E. coli*, *ori*C (Figure 3), and also found in the promoter region of *dnaA*. These GATC- sites are methylated by Dam methylase (see Section 1.3.1). Before initiation of replication, these sequences are fully methylated (Boye, et al. 2000; Mott and Berger 2007). However, the newly synthesized strands contain hemimethylated GATC sequences, that is; only one strand is methylated. The GATC-sites in *ori*C remain hemimethylated for up to one-third of the cell cycle (Campbell and Kleckner 1990) (Figure 5).

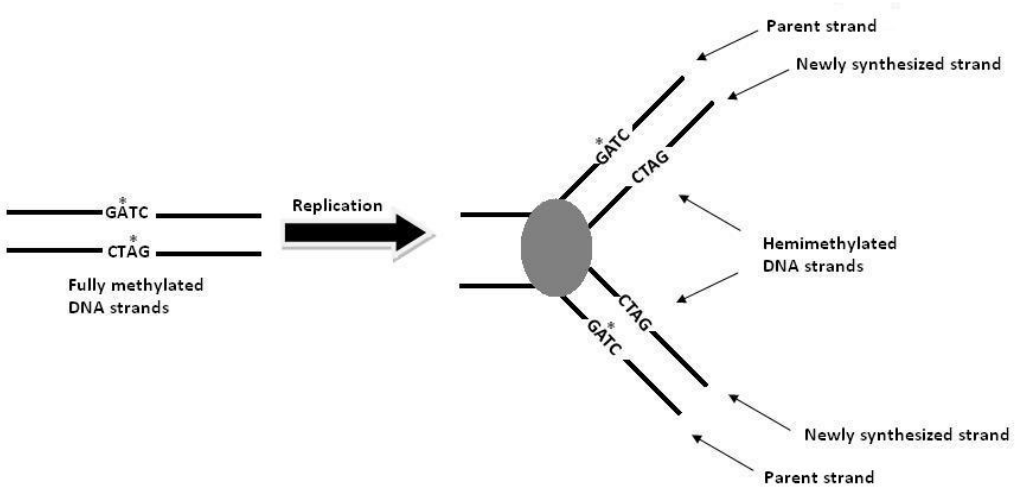

Figure 5: Schematic illustration of methylated and hemimethylated DNA strands during replication. (*) represents methylated adenine at N^6 position.

Fully methylated DNA strands are required to initiate a new round of replication *in vivo*; however, this is not the case in initiation *in vitro.* Initiation can occur on hemimethylated and unmethylated origins *in vitro* (Messer, et al. 1985; Boye 1991). It has been concluded that an intracellular factor inhibits initiation of replication in hemimethylated origins *in vivo*. This factor is identified and known as the SeqA protein (von Freiesleben, et al. 1994; Lu, et al. 1994) (see Section 1.4).

1.3.4.2 Inactivation of DnaA

A process termed regulatory inactivation of DnaA, RIDA, regulates inactivation of ATP-DnaA. This regulatory process requires two proteins; the first one is β-clamp (β subunit of DNA polymerase III holoenzyme), which is part of the replisome complex (Katayama, et al. 1998). The second protein is Hda, which is a member of the AAA+ family proteins that may interact with and hydrolyze ATP (Neuwald , et al. 1999; Kato and Katayama 2001). The Hda and β-clamp form a complex that convert ATP-DnaA to its inactive form (ADP-DnaA) by stimulating the ATP hydrolysis (Kato and Katayama 2001).

1.3.4.3 Titration of DnaA

Titration of DnaA protein is found to occur outside *ori*C. This mechanism involves a site termed *datA* that contain five DnaA-boxes. DnaA binds to the *datA* locus with exceptionally high affinity (Kitagawa, et al. 1996; Kitagawa, et al. 1998). In addition to the DnaA boxes, the *datA* locus contains a single binding site for the IHF protein that stimulates the binding of DnaA molecules (Kitagawa, et al. 1996).

The *datA* locus titrates unusually high amount of DnaA, and makes it difficult to reach the threshold level of DnaA required for the initiation reaction (Messer 2002). *datA* is located near *ori*C, and once *datA* is replicated it titrates twice as much DnaA molecules, resulting in extremely reduced DnaA level at this point in the cell cycle (Boye, et al. 2000).

1.4 The SeqA protein

The *Escherichia coli* SeqA protein is a negative modulator of chromosomal replication and was first discovered in a genetic screen for mutants that would allow initiation of hemimethylated origins in *dam*$^-$ strains (von Freiesleben, et al. 1994; Lu, et al. 1994 ; Waldminghaus 2009).

1.4.1 Structure of SeqA

SeqA is a 21 kDa protein which is composed of 181 amino acids (Lu, et al. 1994) and forms two functional domains; the N- terminal domain (residues 1-33) (Guarné, et al. 2005; Odsbu, et al. 2005) and the C-terminal domain (residues 64-181) (Guarné, et al. 2002; Guarné, et al. 2005).

The C-terminal domain of SeqA folds into three antiparallel β-sheets and seven α-helices (Figure 6). This SeqA domain was found to be the DNA-binding domain as it forms a specific complex with hemimethylated (and methylated) DNA (Guarné, et al. 2002; Fossum, et al. 2003; Fujikawa, et al. 2003).

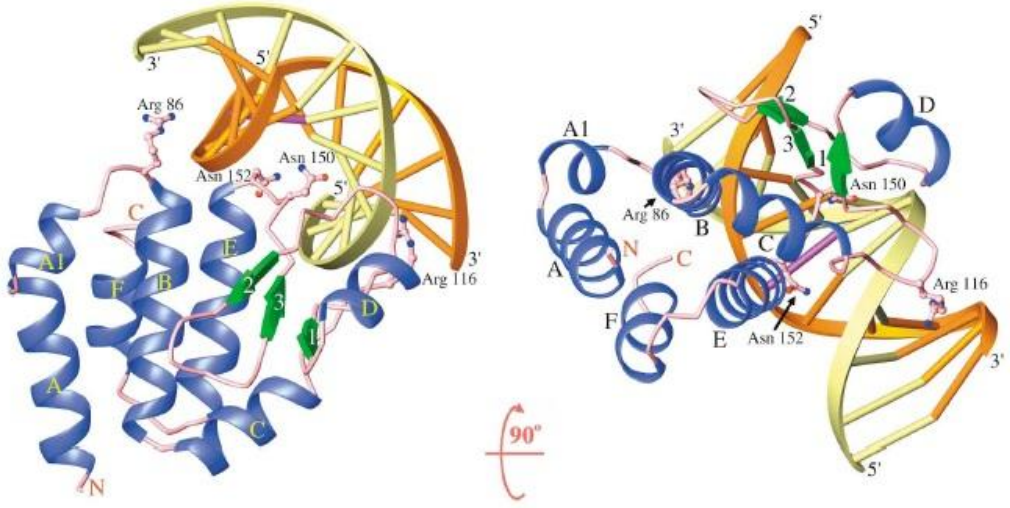

Figure 6: The SeqA-C terminal. Ribbon diagram of C-terminal domain of SeqA protein bound to hemimethylated DNA. It consists of three-stranded antiparallel β-sheets showed in green (β1, β2 and β3) and seven α-helics showed in blue (A, A1, B, C, D, E and F). This figure is from (Guarné, et al. 2002).

The N-terminal domain of SeqA folds into three secondary structures, one β-strand and two α-helices (α1 and α2) (Figure 7). The N-terminal domain of SeqA has been shown to have multimerization activity that causes the formation of the SeqA multimers by mediating the SeqA-SeqA interaction (Odsbu, et al. 2005). This multimerization activity might be involved in forming a large SeqA-DNA cluster on hemimethylated *ori*C, which prevent reinitiation of

replication (Fujikawa, et al. 2003; Odsbu, et al. 2005). In additional studies, it was observed that the N-terminal domain is responsible for the dimer formation between SeqA molecules (Guarné, et al. 2005; Odsbu, et al. 2005), and also multimer formation by hydrophobic interaction between dimer-molecules (Guarné, et al. 2005).

The N-terminal domain is also found to form a filament structure of dimers that is important for SeqA's function in synchronization of DNA replication (Odsbu, et al. 2005). These findings indicate that DNA replication might be asynchronous in *seqA* mutants lacking filament formation property (Odsbu, et al. 2005).

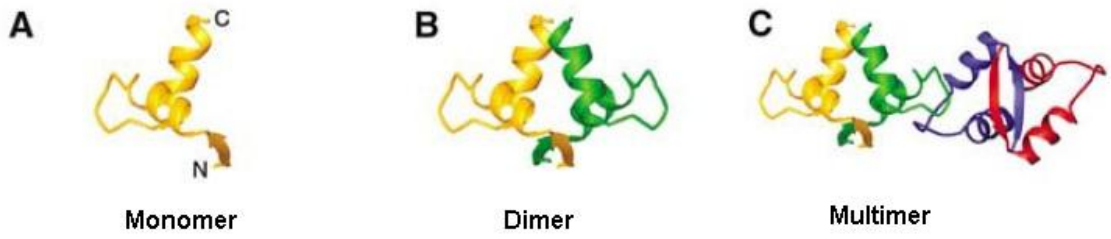

Figure 7: The SeqA-N terminal. Ribbon diagram of (A) single SeqA-N subunit in yellow, (B) two SeqA-N subunit in yellow and green and (C) two dimer subunits, or multimer, in yellow, green, blue and red. This figure is modified from (Guarné, et al. 2005).

A flexible linker of 28 residues is found to separate the N-terminal domain and the C-terminal domain of SeqA protein (Guarné, et al. 2002; Guarné, et al. 2005) (Figure 8). *In vitro* studies has shown that *seqA* mutants with changes in the flexible linker affects the ability of multimerization of SeqA molecules (Kang, et al. 2007).

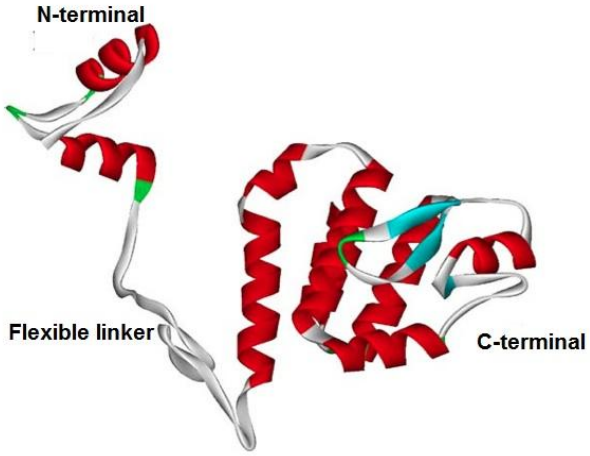

Figure 8: The SeqA protein structure. The C-terminal domain is the DNA-binding domain, and the N-terminal is responsible for the multimer formation between SeqA dimers. A flexible linker binds the C- terminal and the N-terminal together. This figure is from (Daghfous, et al. 2009).

1.4.2 Binding of SeqA to DNA

The C-terminal domain of SeqA is the DNA binding domain. SeqA binds as a dimer to two GATC sequences (Brendler and Austin 1999; Brendler, et al. 2000; Han, et al. 2004) that are separated by up to 31 base pairs (Brendler, et al. 2000) (Figure 9). The SeqA protein binds to the newly replicated origins containing hemimethylated GATC sites (Slater, et al. 1995). Experiments have shown that the ability of SeqA to oligomerize is important for its' activity in preventing reinitiation of replication (Odsbu, et al. 2005). Due to self-association activity of SeqA, SeqA is capable of forming a cluster complex with DNA at the *ori*C region after replication (Hiraga, et al. 1998; Onogi, et al. 1999). This cluster forming is shown to be essential for function of SeqA in preventing reinitiation (Fujikawa, et al. 2003).

GATC sites in *ori*C are, however, not the only chromosome sites SeqA protein binds to. There are approximately 4000 GATC sites distributed around the *E. coli* chromosome that become hemimethylated during replication (Guarné, et al. 2005). Most of SeqA molecules are therefore found to bind to GATC outside the *ori*C (Guarné, et al. 2005).

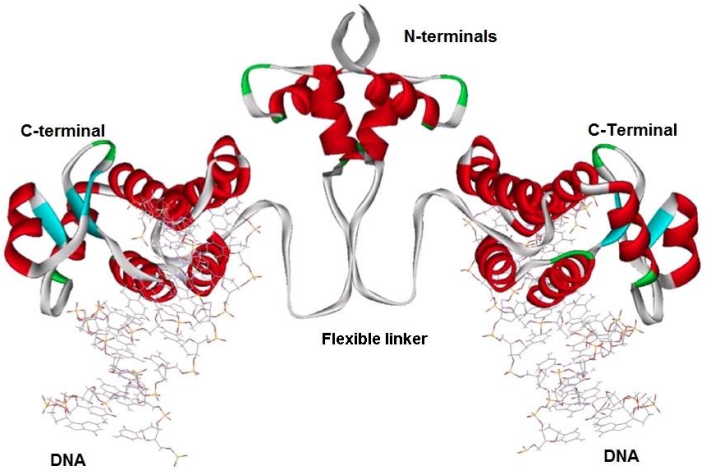

Figure 9: Binding of SeqA to DNA. SeqA molecules bind to DNA by the C-terminal domain, while the N-terminal domains create dimers (and multimers) of SeqA molecules. A flexible linker separates the C- and N-terminal domains. This figure is modified from (Daghfous, et al. 2009).

In addition, immunofluorescence microscopy showed that SeqA protein form foci *in vivo* (Odsbu, et al. 2005), which represents SeqA multimers behind the replication forks. Figure 10 shows how SeqA molecules are suggested to bind to the DNA behind the replication fork.

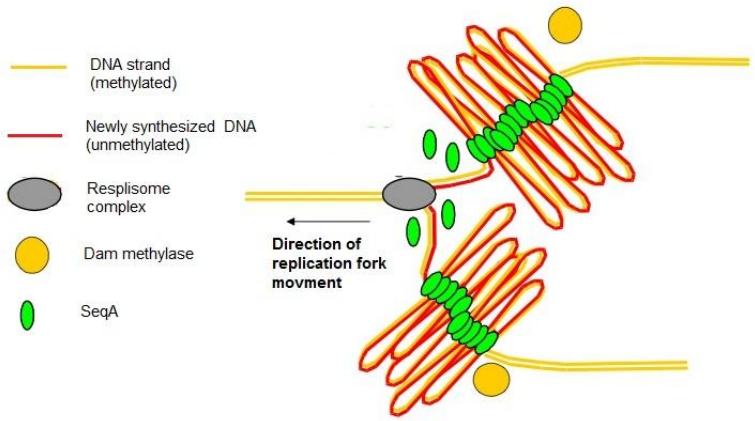

Figure 10: Binding of SeqA to the newly replicated, hemimethylated DNA strands behind the replication fork. This figure is drawn by Kirsten Skarstad.

1.4.3 Properties of SeqA

1.4.3.1 SeqA prevents reinitiation of replication

SeqA was identified as an important factor involved in sequestration (see Section 1.3.4.1), and its role is to bind specifically to the hemimethylated GATC sequences in *ori*C and prevent reinitiation for about 1/3 of the cell cycle (Campbell and Kleckner 1990). Overinitation has been observed in cells lacking SeqA protein, where the newly synthesized origins are reinitiated (von Freiesleben, et al. 1994; Lu, et al. 1994 ; Slater, et al. 1995).

SeqA binds to the left end of the *ori*C region at the AT rich region (Figure 3), where the initial strand separation occurs and thereby inhibits formation of the open complex (Torheim and Skarstad 1999). SeqA bind to the newly synthesized hemimethylated DNA strands and keeps all origins inactivated for one third of the cell cycle (Campbell and Kleckner 1990). In this period of time, dam methylase is unable to methylate the newly synthesized GATC sites. It is yet not known how sequestration ends, but dissociation of SeqA and methylation of the hemimethylated DNA strands by Dam methylase is required (Waldminghaus 2009).

In addition to sequestration of *ori*C, reinitiation is also prevented by sequestration of the *dnaA* gene promoter (Campbell and Kleckner 1990), that lies 42 kb away from *ori*C (Skarstad and Boye 1994). Inhibition of transcription of the *dnaA* gene prevents DnaA-synthesis during the period of sequestration.

In vitro studies have shown that binding of SeqA to hemimethylated *ori*C specifically blocks the initiator protein, DnaA, from binding to three of its binding sites in *ori*C. Another experiment with supercoiled *ori*C have shown that SeqA was able to prevent strand separation by DnaA (Torheim and Skarstad 1999). Blocking DnaA from its binding sites and preventing strand separation might affect formation of the initial complex in replication (see Section 1.3.3).

1.4.3.2 SeqA influences DNA topology

Studies have shown that, in addition to its role in sequestration, SeqA has other functions. In a study to investigate how SeqA prevent the replication, it was found that SeqA also has an effect on DNA topology by restraining the negative supercoiled DNA (Torheim and Skarstad 1999). These findings were confirmed by another study, where the *seqA4* mutant was studied.

The results showed that the *seqA4* mutant failed to restrain the negative supercoiled DNA (Odsbu, et al. 2005) (Figure 11).

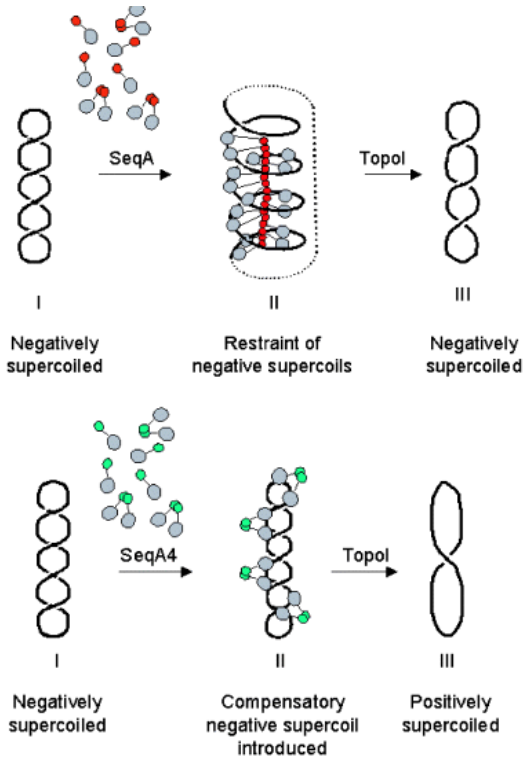

Figure 11: Influence of SeqA on DNA topology. SeqA restrain the negative supercoils, preventing TopoI from forming the positive supercolis. However, *seqA4* mutant lack this property allowing TopoI to form positive supercoiled conformation. This figure is from (Odsbu, et al. 2005).

1.4.3.3 The role of SeqA in chromosome segregation and organization

In studies where SeqA was overexpressed, it was observed inhibition of segregation of nucleoids, which may delay cell division This observation suggested that SeqA might have a role in chromosome segregation (von Freiesleben, et al. 2000; Bach, et al. 2003). However, segregation was not affected in an earlier experiment where the *seqA* gene was deleted. It was therefore suggested that other factors were involved in segregation (Lu, et al. 1994). Other studies have shown that cells with SeqA null mutant have abnormal nucleoid distribution, and a higher frequency of anucleoid cells (Onogi, et al. 1999).

In light of these results, it is suggested that SeqA may have an important role in segregation of sister chromosomes, as well as in organization of newly replicated DNA at replication forks (Onogi, et al. 1999; Brendler, et al. 2000; Hiraga, et al. 2000).

1.4.3.4 SeqA binds to Topoisomerase IV (Topo IV)

In a study where a bacterial two-hybrid system was used, it was revealed that SeqA binds to ParC; the subunit of Topo IV (topoisomerase IV) (Kang, et al. 2003). Topo IV is essential for chromosome segregation by catalyzing the decatenation of daughter chromosomes (Kato, et al. 1992). It has also been shown that Topo IV activity is depended on SeqA-DNA binding strength (Kang, et al. 2003). Topo IV was introduced to different forms of DNA, and showed to be most efficient in converting hemimethylated DNA to a relaxed form. This indicates that SeqA might have a role in segregation by stimulating the decantation activity of Topo IV (Kang, et al. 2003).

1.4.4 Localization of the SeqA protein in the cell

Experiments involving SeqA tagged with GFP (Green fluorescent protein) or immunolabeled with SeqA-antibodies has been conducted. These experiments were able to detect SeqA's location in the cell, and revealed SeqA as foci at the replication forks (Brendler, et al. 2000; Hiraga, et al. 2000; Fossum, et al. 2003; Molina and Skarstad 2004). These results were confirmed in another study using global ChIP on Chip analysis of SeqA (Waldminghaus, et al. 2012). The results showed that SeqA binds to DNA behind the replication forks during replication, with more frequent binding at new replication forks, and less frequent binding at old replication forks. It was suggested that SeqA from the old forks is possibly transferred to the new forks (Waldminghaus, et al. 2012).

1.4.5 SeqA mutations

Von Freiesleben et al. (1994) have isolated several *seqA* mutants. The mutations were localized in the N-terminal domain (*seqA4*), the central part (*seqA3*) and the C-terminal domain (*seqA2*) (Fossum, et al. 2003). In the *seqA2* mutant the amino acid asparagine in the position 152 is replaced by aspartic acid in the C-terminal domain. In the *seqA4* mutant, the amino acid alanine in the position 25 is replaced by threonine in the N-terminal domain (von Freiesleben, et al. 1994).

The *seqA2* mutant lacks the ability to bind to hemimethylated DNA, and *in vivo* studies has shown that *seqA2* is inactive in sequestration (Fossum, et al. 2003). This observation is related to the inability of *seqA* mutant protein to bind to the hemimethylated *ori*C and prevent reinitiation (Fossum, et al. 2003) (see Section 1.4.3.1).

The point mutation in the *seqA4* mutant has shown to affect function of SeqA in multimerization and synchrony of initiation of DNA replication. Gel filtration study showed that *seqA4* mutant protein could form dimers, but not multimers (Odsbu, et al. 2005). It was therefore suggested that a dimer is the basic binding unit to hemimethylated DNA. It has also been shown that high levels of SeqA4 protein resulted in restored synchrony in a Δ*seqA* strain *in vivo* (Odsbu, et al. 2005). These observations suggest that sequestration does not necessarily require the formation of SeqA- multimers, as long as the local concentration of the SeqA proteins is high enough.

Immunofluorescence microscopy has shown that wild type SeqA forms foci behind the replication forks. The foci represent SeqA molecules as they bind to the newly replicated, hemimethylated DNA behind the replication forks. However, the *seqA4* mutant have shown to be unable of forming foci (Odsbu, et al. 2005). These results indicate that the *seqA4* mutant is not capable of forming stabilized structure of the SeqA protein behind the replication forks as a wild type does.

1.5 The RecA protein

RecA is a multifunctional protein in *E.coli* encoded by the *recA* gene. The RecA protein plays an important role in several biological processes, such as DNA repair, homologous recombination and induction of the SOS response. The RecA protein consists of 352 amino acids and has both DNA-dependent ATPase and ATP-dependent DNA binding activities. RecA is a central protein in all homologous recombination events in *E. coli* where it pairs two homologous DNA molecules. RecA forms a nucleoprotein filament, which is the active mediated DNA strand exchange. The filament is composed of DNA and RecA that assembles to both single- and double stranded DNA (ssDNA and dsDNA), and promotes strand exchange in homologous DNA recombination. (Friedberg, et al. 2006)

RecA also plays an important role in regulation of the SOS response in *E. coli*. Intracellular signals for SOS induction are initiated when the genome of an *E. coli* cell is damaged. Those signals are found to be generated from regions of ssDNA when a damaged DNA template is about to be replicated or when the DNA replication is interrupted. The RecA filament is then formed by binding of RecA to the ssDNA regions. The RecA-ssDNa filaments are able to interact with the LexA protein resulting in proteolytic cleavage of LexA. LexA is a protein involved in the SOS response, by acting as a repressor of SOS-regulating genes, such as *recA*. When LexA protein is cleaved, *recA* and other SOS genes are expressed. Once the damaged DNA is recovered, the amount of RecA protein present in the RecA-ssDNA filament decreases, meanwhile the synthesis of LexA protein increases leading to repression of the SOS reopens. (Friedberg, et al. 2006)

1.5.1 *recA* mutants

recA mutants have shown to be deficient in homologous recombination. However, it has been shown that strains of *E. coli* with *recA* mutation are still able to survive, only with lower viability. This observation is suggested to be related to less frequent chromosomal fragmentation in *E.coli*. In a study, Kouzminova et al. (2004) searched for *recA*-depended *E.coli* mutants by using a color screen. The color screen involved using *lacZ recA* mutants of *E.coli* having $lacZ^+$ $recA^+$ plasmids with a temperature-sensitive origin of replication, plated on MacConkey-lactose agar. Purple colonies at 28°C indicated $recA^+$ strains, while pale colonies at 42°C indicated $recA^-$ strains. To screen for *recA-dependent* mutants in this study, the colonies were grown at 34°C, which make the cell loose the RecA plasmids at low rate. Two types of colonies were obtained at 34°C, sectored colonies which were not *recA*-

dependent and could therefore grow in the absence of *recA*, and solidly colored medium- or small-sized colonies indicated *recA-dependent* mutants not able to grow without the *recA*$^{+}$ gene.

Several mutants were found to be *recA-dependent*; one of mutants was *seqA*, with an unknown type of mutation. In order to confirm and characterize the mutants obtained in this study, Kouzminova et al. constructed a *ΔseqA ΔrecA* strain. This strain oddly was observed to gain a cold-sensitive property as it was barely viable at 45°C and not viable at temperatures bellow 34°C (Figure 12).

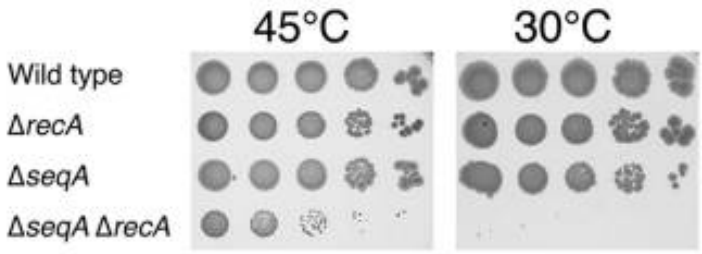

Figure 12: Colony formation of a wild type *E. coli*, a *ΔrecA* mutant, a *ΔseqA* mutant and a *ΔseqA ΔrecA* mutant at 45°C and 30°C. This figure is form (Kouzminova, et al. 2004).

1.6 Aim of the study

The SeqA protein plays an important role in regulation of replication, by preventing reinitiation of replication of the newly synthesized strands. SeqA protein is also found to organizing newly synthesized DNA. SeqA protein consists of two functional domains; the C-terminal DNA binding domain and the N-terminal multimerization domain. The *seqA4* mutant protein has point mutation where the amino acid alanine is replaced with threonine in position 25. This makes the *seqA4* mutant protein unable to multimerize as wild type SeqA does.

The aim of this study is to search for interacting partners for the SeqA protein by using genetic screening as a tool. The SeqA interacting partners are assumed to bind to SeqA and help promoting its function. In order to perform the screen, a genomic library was constructed by ligating *E. coli* genomic DNA fragments to a multicopy plasmid, and transformed into an *E.coli* SF146 strain with double mutation; *seqA4 and ΔrecA.* This double mutant strain was constructed on basis of findings from Kouzminova et al. where *ΔseqA ΔrecA* strain showed to be viable at 45°C, but not bellow 34°C (see Section 1.5.1). Due to the purpose of discovering new proteins that *bind* to SeqA protein, a strain with dysfunctional SeqA protein was used in this study and not *ΔseqA*. The double mutation of SF146 is shown be viable at 42 °C but not at 30°C (Solveig Fossum-Raunehaug, unpublished).

We selected for transformation mutants that would allow SF146 to grow at 30°C. These transformants may contain a gene encoding a protein that interact with SeqA4 protein and promote its function. These colonies were selected, plasmids were purified and digested with restriction enzymes. The presences of DNA fragments were analyzed with agarose gel electrophoresis.

2 MATERIALS

2.1 Bacterial strains and plasmids

Table 1: Bacterial strain used in this study

Bacterial strain	Genotype	Source
MG1655	F^- λ^- *rph-1*	(Guyer, et al. 1981)
MC1000	F^- *ara*D139, Δ*(ara, leu)*$_{7697}$, Δ*lac X*74, *gal*U, *gal*K, *str*A	(Casadaban and Cohen 1980)
CM735	*met*E46 *trp*-3,*his*-4,*thi*-1,*gal*K2,*lac*Y1 or *lac*Z4,*mth*-1 *ara*-9,*tsx*-3,*ton*-1,*rps*L8 or 9,*sup*E44 λ^-	(Hansen and von Meyenburg 1979)
One Shot® OmniMAX™ 2 $T1^R$	F′ {*pro*AB$^+$ *lac*I^q *lac*ZΔM15 *Tn*10(Tet^R) Δ(*ccd*AB)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80(*lac*Z)ΔM15 Δ*(lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D	Inivtrogen®
One Shot® TOP10	F- *mcr*A Δ*(mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 *Δlac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)$_{7697}$ *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G	Invitrogen®

Bacterial Strain	Relevant features	Source
ALO382	MC1000 *dam*-/pUN121	(Nilsson, et al. 1983)
ALS972	MG1655 *rec*A938::cat	(Winans, et al. 1985)
SF122	CM735 *seqA4 zbf*-3057::*Tn10*	Solveig Fossum-Raunehaug (unpuplished)
SF146*	SF122 (CM735 *seqA4-zbf*-3057::*Tn10*) *recA938::cam*	Solveig Fossum-Raunehaug (unpuplished)
One Shot® OmniMAX™ 2 $T1^R$/pUN121	One Shot® OmniMAX™ 2 $T1^R$/pUN121	This study
K1	SF146/pUNK1	This study
K2	SF146/pUNK2	This study

KK1	SF146/pUNKK1	This study
KK2	SF146/pUNKK2	This study
KK3	SF146/pUNKK3	This study
KK4	SF146/pUNKK4	This study
KK5	SF146/pUNKK5	This study
KK6	SF146/pUNKK6	This study
KK7	SF146/pUNKK7	This study

*P1 ALS972 x SF122

Table 2: Plasmids used in this study

Plasmid	Marker	Source
pUN121	Ampicillin	(Nilsson, et al. 1983)
pUC19	Ampicillin	Invitrogen®
pUNK1	Ampicillin	This study
pUNK2	Ampicillin	This study
pUNKK1	Ampicillin	This study
pUNKK2	Ampicillin	This study
pUNKK3	Ampicillin	This study
pUNKK4	Ampicillin	This study
pUNKK5	Ampicillin	This study
pUNKK6	Ampicillin	This study
pUNKK7	Ampicillin	This study

2.2 Antibiotics

Table 3: Antibiotics used in this study

Antibiotic	Stock solution	Producer
Ampicillin	100 mg/ml	Bristol-Meyers Squibb
Tetracycline	5 mg/ml	Arcopharma AS
Chloramphenicol	30 mg/ml	Sigma-Aldrich ®

2.3 Enzymes

Table 4: Enzymes used in this study

Enzyme	Buffer	Source
EcoRI	NEBuffer 4	New England Biolabs® Inc.
Proteinase K		Sigma-Aldrich ®
Antarctic acid phosphatase	Antarctic acid phosphatase buffer	New England Biolabs® Inc
HindIII	NeBuffer 2	New England Biolabs® Inc
RNase		Sigma-Aldrich ®
T4 DNA ligase	T4 DNA ligase buffer	New England Biolabs® Inc.

2.4 Chemicals

Table 5: Chemicals used in this study

Chemical	Producer
Acetic acid	VWR®
Agarose	Sigma-Aldrich ®
Bromophenol Blue	Sigma-Aldrich ®
$CaCl_2$ (Calcium chloride)	Sigma-Aldrich ®

Chloroform	Sigma-Aldrich ®
EDTA (ethylene-diamine-tetra-acetat)	Sigma-Aldrich ®
Ethanol	Kemetyl Norge AS
GelRed	Life Technologies
Glyserol	Sigma-Aldrich ®
Isopropanol (2-propanol)	Sigma-Aldrich ®
NaCl (sodium-chloride)	Merck®
Methanol	Merck®
Sucrose	VWR®
Phenol	Sigma-Aldrich ®
SDS (sodium-dodecyl-sulfate)	BioRad
Tris (Hydroxymethyl aminomethan)	Sigma-Aldrich ®

2.5 Equipment and apparatus

Table 6: Equipment and apparatus used in this study

Equipment	Model	Producer
NanoDrop ™	ND-1000 Spectrophotometer	Thermo Science
UV Spectrophotometer	UV1800	Shimadzu
Gel Electrophoresis Apparatus	Power PAC 300	BIO-RAD
Radiometer analytical (pH-meter)	Inolab	WTW
Refrigerated Microcentrifuge	5417 R	Eppendorf
Microsentrifuge	Mikro 200R	Hettich
Refrigerated Centrifuge	5930	KUBOTA
Microwave	Wavedom	LG
Heat block	QBD2	Grant Instrument

Spectrophotometer cuvette	Semi-micro	BrandTech Scientific ®
Shaking incubator	Thermomixer comfort	Eppendorf
Pipette tip	0,2-1000 µl 0,1-200 µl	Eppendorf Ranin
Needle	Mod 3	BD microlance
Pipette	0,2-2 µl 0,5-5 µl 2-20 µl 10-100 µl 100-1000 µl	Thermo Scientific
Glass pipette	2 ml 5 ml 10 ml 25 ml 50 ml	Sarstedt
Ultracentrifuge	Optima L-90K	Beckman Coulter
Vacuum suction		Laboratory creation
Vortexer	MS3 digital	IKA Works, Inc,
Deep Freezer	ULT Freezer, -86^{o}C	Thermo Scientific
Liquid nitrogen	-	Laboratory stock
Liquid nitrogen container	-	Isotherm
Round bottom tube	BD Falcon plypropylene	Flacon ®
Ultracentrifuge rotor	SW28 SW 41	Beckman
Electroporation apparatus	Gene pulser ® II	BIO-RAD
Electroporation cuvette	0,2 cm gap	BIO-RAD
Bunsen burner	FIREBOY plus	IBS - INTEGRA Biosciences

2.6 Commercial kits

Table 7: Commercial kits used in this study

Kit	Producer
JETstar (midi-prep): The novel plasmid purification system	Promega
QIAprep Spin Miniprep Kit	QIAGEN
Wizard®Genomic DNA Purification Kit	Genomed

2.7 Buffers, solutions and media

Table 8: Agarose Gel Electrophoresis buffers used in this study

Buffer	Compositions
Agarose gel electrophoresis buffer (TAE)	*50X TAE for 500 ml:* 121 g Tris base (MW=121.1) 28,6 ml acetic acid 50 ml 0.5 M EDTA (pH 8) up to 500 ml dH_2O *1X TAE:* 40 ml 50X TAE up to 2L dH_2O
1% Agarose	1g Agarose up to 100 ml dH_2O
Agarose loading buffer	500 µl dH_2O 500 µl Glycerol 87% 10-20µl bromophenol blue

Table 9: Enzyme buffers used in this study

Buffer	Compositions
Antarctic acid phosphatase buffer	50 mM Bis-Tris-Propane-HCl 1 mM MgCl2 0.1 mM ZnCl2 pH 6.0
NEBuffer 2 (HindIII buffer)	*1X NEBuffer 2:* 50 mM NaCl

	10 mM Tris-HCl 10 mM MgCl2 1 mM Dithiothreitol pH 7.9
NEBuffer 4 (EcoRI buffer)	*1X NEBuffer 4:* 50 mM potassium acetate 20 mM Tris-acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol pH 7.9
T4 DNA ligase buffer	*1X T4 DNA Ligase Reaction Buffer:* 50 mM Tris-HCl 10 mM MgCl2 1 mM ATP 10 mM Dithiothreitol pH 7,5

Table 10: JETstar (midi-prep) buffers used in in this study

Buffer	**Compositions**
E1 solution	50 mM Tris-HCl (pH 8.0) 10 mM EDTA 100 µg/ml RNase A
E2 solution	200 mM NaOH 1%(w/v) SDS
E3 solution	1 M potassium acetate (pH 5.5 with acetic acid)
E4 solution	100 mM Sodium acetate (pH 5.0 with acetic acid) 600 mM NaCl 0.15% Triton X-100
E5 solution	100 mM Sodium acetate (pH 5.0 with acetic acid) 800 mM NaCl
E6 solution	100 mM Sodium acetate (pH 5.0 with acetic acid) 1.500 mM NaCl

Table 11: Other types of buffers used in this study

Buffer	Compositions
TE-buffer (Tris-HCl)	10 mM Tris-Cl, pH 8 1 mM EDTA
Sucrose buffer	1M Nacl 20 mM Tris-HCl (pH 7,5)' 5mM EDTA

Table 12: Growth medium used in this study

Medium	Compositions
LB medium	10 g Tryptone 5 g Yeast extract 10 g NaCl up to 1 l dH_2O
SOC	10 g Tryptone 5 g Yeast extract 0,5 g NaCl 20 mM glucose up to1 l dH_2O

3 METHODS

3.1 Bacterial growth conditions

Different bacterial strains require different growth conditions and nutrients (Madigan, et al. 2008). Bacterial strains used in this study were stored in a glycerol stock (750 µl bacterial culture and 750 µl 87% glycerol) at -80^{o}C. Preparation of a bacterial culture was obtained by inoculating the bacteria from the glycerol stock in an appropriate amount of LB medium at 30°C, 37°C or 42°C with vigorous shaking (~200 rpm) overnight. When needed, ampicillin, tetracycline and/or chloramphenicol were added to a final concentration of 100 µg/ml, 5 µg/ml and 30 µg/ml, respectively.

When a specific OD of the bacterial culture was desired, the overnight culture was first diluted 1:100 in LB medium and incubated with vigorous shaking (~200 rpm). OD_{600} measurements were taken (see Section 3.3) of samples from the diluted overnight culture every ~30 minutes, and more frequently during the exponential phase.

3.2 Media

In order to obtain bacterial growth in the laboratory it is necessary to use a growth medium containing essential nutrient such as amino acids and glucose. There are different types of medium with different content (Madigan, et al. 2008). In this study SOC- and LB medium were used. LB was used in both liquid and solid form.

3.2.1 LB

LB is a widely used rich medium in both liquid from (Lurina-Bertani broth) and solid form (LB-agar). Both liquid and solid forms contain tryptone, yeast extract, NaCl and distilled water and are adjusted to pH 7 with NaOH (Table 12). To prepare solid LB, 1,5 % agar is added before sterilization. LB-agar plates were prepared by melting the LB- agar medium and pouring ~ 25 ml of the melted medium into sterile plastic plates and let it solidify before use (Sambrook and Russell 2001).

3.2.2 SOC

SOC is another rich medium that contains tryptone, yeast extract, NaCl and distilled water (Table 12). In addition, SOC medium contains 20 mM glucose that makes the medium even richer than LB (Sambrook and Russell 2001).

3.3 Measurement of bacterial density, OD_{600}

In assays where a specific density of bacterial culture was required, OD measurements were taken at 600 nm by using UV-spectrophotometer (OD_{600}). UV-spectrophotometer is an instrument that passes light through a cell suspension and detects the unchartered light that emerges and gives reading in optical density units (Madigan, et al. 2008).

3.4 Serial dilution

Low concentrations of DNA, bacterial cultures etc. were needed for a number of assays and serial dilution was therefore used. The purpose of serial dilution for a bacterial culture is to obtain only a few viable cells that would be countable after plating and incubation. Figure 13 shows an example scheme of a serial dilution of bacterial culture.

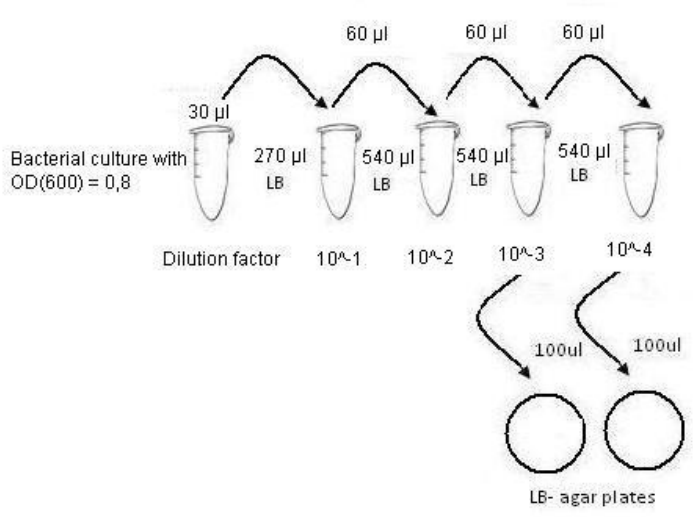

Figure 13: Scheme of serial dilution. In order to get countable number of colonies on LB-agar plates from a bacterial culture of OD_{600} 0,8.

The number of countable colonies is expected to appear in the last two tubes and it is therefore often chosen to plate 100 µl from the last tubes and incubate. The number of colonies that would appear is then used to calculate the number of viable cells in the original culture. Serial dilution was also used to dilute concentrated DNA.

3.5 DNA techniques

3.5.1 Purification of plasmid DNA

3.5.1.1 Large scale

Purification of plasmids involve growth of bacterial culture, harvesting, lysis of the bacteria and purification of the plasmid DNA (Sambrook and Russell 2001). Several plasmids for this study were purified in large scale using JETstar plasmid purification midi kit from Genomed to obtain 0,2-1µg DNA. JETstar plasmid purification system is a unique anion exchange resin. The procedure employs a modified alkaline/SDS method to prepare the cleared lysate. Overnight culture (100 ml) was centrifuged and the pellet was resuspended with 4 ml solution containing RNase (E1 solution). The cells were afterwards treated with 4 ml solution containing 1% SDS (E2 solution) to lyse the cells, followed by neutralizing with 4 ml solution containing 1 M potassium acetate with pH 5,5 (E3 solution). After neutralization, the lysate was applied onto an equilibrated (with 10 ml E4 solution) JETSTAR column, where the plasmid DNA was bound to the anion exchange resin. The column was then washed twice with 10 ml E5 solution containing sodium acetat (pH 5) and NaCl removing RNA and all other impurities. Next, the purified plasmid DNA was eluted with E6 solution and precipitated with 3,5 ml isopropanol and centrifuged for 30 minutes at 4°C, 12000 rpm. The plasmid was then washed with ~200 µl 70% ethanol and centrifuged for ~5 minutes at 4°C, 12000 rpm. After removing all of the ethanol, the pellet was air dried for ~10 minutes and dissolved with 100 µl TE-buffer or water. The concentration of DNA was measured by using NanoDrop 1000 (see Section 3.5.3).

3.5.1.2 Small scale

Several plasmids were also purified in smaller scale using QIAprep Spin Miniprep Kit from QIAgen to obtain up to 20 µg plasmid DNA. Purification of plasmids using QIAgen miniprep involves: preparation and clearing of a bacterial lysate, adsorption of DNA onto the QIAprep membrane and washing and elution of plasmid DNA.

Overnight culture (1-5 ml) was centrifuged and the pellet was resusupended with 250 µl solution containing RNase (Buffer P1). Next step was lysis of the cells by adding 250 µl Buffer P2, inverting the tube 4-6 and then incubation for not more than 5 minutes. The bacterial lysate was then washed by adding 350 µl Buffer N3 and mixed immediately by inverting the tube 4-6 times. The washed lysate was then centrifuged for 10 minutes at room temperature and 13 000 rpm. The supernatant was applied to the QIAprep spin column and centrifuged for 30-60 seconds with 13 000 rpm and the flow-through were discarded. Next, the column was washed with 500 µl Buffer PB to remove any trace of nuclease activity and centrifuged for 30-60 seconds same as earlier. The column was washed further with 750 µl Buffer PE and centrifuged twice for 30-60 seconds. Finally, the column was placed in a new microcentrifuge tube and 50 µl Buffer EB or water was added to the column and let stand for 1 minute before centrifugation for 1 minutes with 13 000 rpm to elute the plasmid DNA. The concentration was determined with NanoDrop 1000 (see Section 3.5.3) and the DNA solution was either immediately used for assays such as gel electrophoresis or stored at -20ºC (see Section 3.5.5).

3.5.2 Purification of genomic DNA

Purification of genomic DNA involves: lysis of cells, removal of proteins and contaminants, washing and resuspension of the DNA. Wizard® Genomic DNA Purification Kit from Promega was used for this purpose.

Overnight culture (25 ml) was pelleted by centrifugation for 2 minutes at 24°C, 8500 rpm. 600 µl Nuclei Lysis solution was then added to break down the cell walls and membrane and to release the DNA and other intracellular components. The cell culture containing Nuclei Lysis solution was incubated at 80°C for 5 minutes to ensure lysis of the cells. The lysate was then cooled to room temperature and 3 µl RNase was added and mixed by inverting the tube 3-5 times, followed by incubation at 37°C for 15-60 minutes. Afterwards, proteins were then removed by adding 200 µl Protein Precipitation Solution and vortexed for ~20 seconds before incubating on ice for 5 minutes. The mixture was then centrifuged for 3 minutes at 4°C, 8500

rpm. The supernatant containing DNA was transferred to a new centrifuge tube to precipitate by adding 600 µl isopropanolol and gently mix until thread-like strands of DNA formed a visible mass. The DNA was then pelleted by centrifuging for 2 minutes at 24°C, 8500 rpm. Next step involved washing the pellet with 600 µl of room temperature 70% ethanol followed by centrifugation as in previous step. Finally, after removing all of the alcohol and air drying for 10-15 minutes, 100 µl DNA Rehydration Solution was added and the DNA was rehydrated by incubating at 65°C for 1 hour. Alternatively, DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C. Concentration of DNA was determined with NanoDrop 1000 (se Section 3.5.3).

3.5.3 Determination of the concentration of DNA

The concentration of plasmid DNA and genomic DNA was measured by using an UV/VIS spectrophotometer, NanoDrop 1000. The spectrophotometer measures the concentration by measuring the absorbance of nucleic acids at 260 nm wavelength (NanoDrop 1000 Spectrophotometer V3.7 User's Manual). Samples of ~1,5 µl was applied to the apparatus and concentrations were shown in ng/µl.

3.5.4 Dephsphorylation of plasmid

Dephosphorylation of plasmid DNA involves removing of the terminal 5'- phosphate group, which is necessary step before a ligation reaction (see Section 3.6.5). Dephosphorylation prevents self ligation of plasmid DNA, which otherwise would leads to ineffective ligation of the insert DNA to the plasmid (Sambrook and Russell 2001).

Plasmid DNA was dephosphorylated by treating the plasmid with antarctic phosphatase. The reaction was added 1X antarctic phosphatase buffer (1/10 of the reaction volume), and incubated for 15 minutes at 37^{o}C and inactivated by incubating for 5 minutes at 65^{o}C.

3.5.5 Storage conditions of DNA samples

After purifying plasmid and genomic DNA, the samples were stored eithrt at 2–8 ° C for 24 h or at –20°C for longer periods.

3.6 Construction of DNA library

3.6.1 Digestion of DNA with restriction enzyme

In order to construct a genomic library, the selected vector and genomic DNA were first cut with restriction endonuclease enzyme (RE). RE are bacterial enzymes with a biological function to protect the host genome against foreign DNA, such as virus genomes (Madigan, et al. 2008). RE recognizes specific sequence within DNA and cut DNA molecules into fragments that range in length from a few hundred to a few thousands base pairs (Madigan, et al. 2008).

EcoRI is a type II restriction endonuclease that recognizes and cleaves DNA within the recognition sequences (Figure 14). EcoRI cleave their DNA recognition sequences at positions that are staggered by four base pairs, producing 5' overhanging ends (Sambrook and Russell 2001).

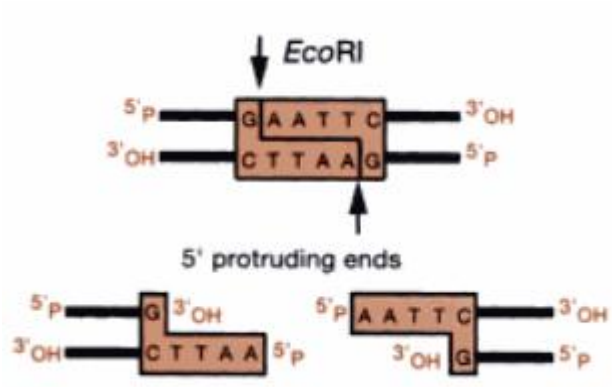

Figure 14: EcoRI recognition site. The arrows indicate the cleavage sites. Note that the sticky ends of the cut sequences have the same sequences if both are read 5' to 3' or 3' to 5'. This figure is from (Sambrook and Russell 2001).

The DNA that is going to be inserted into the plasmid must bear compatible termini, which will allow recognizing and ligating to the plasmid (Sambrook and Russell 2001).

Genomic DNA and plasmid DNA (vector) were cut with EcoRI enzyme and mixed with NEBuffer 4 (1/10 of the reaction volume). The reaction was incubated at 37^{o}C for 2 hours to ensure optimal digestion, and inactivated by incubating at 65^{o}C for 10 minutes.

3.6.2 Sucrose gradient

Sucrose gradient separation is a method that involves separating different DNA fragments according to their sizes. 10-40% sucrose gradient was prepared by transferring ~5,5 ml 40% sucrose solution first, then ~5,5 ml 10% sucrose to a SW-41 tube and inverting by using BioComp GRADIENT MASTER with the following settings: time 1:48, angle 81,5, speed 17. EcoRI-cut genomic DNA solution was then layered on top of the sucrose gradient and ultracentrifuged for 24 hours at 4°C, 22 000 rpm to separate the fragments along the gradient. After ultracentrifugation, the fragments were separated in different layers according to size. The heavier fragments layed near the bottom, while the small fragments layed near the top. Figure 15 summarizes the steps in separating DNA fragments using 10-40% sucrose gradient.

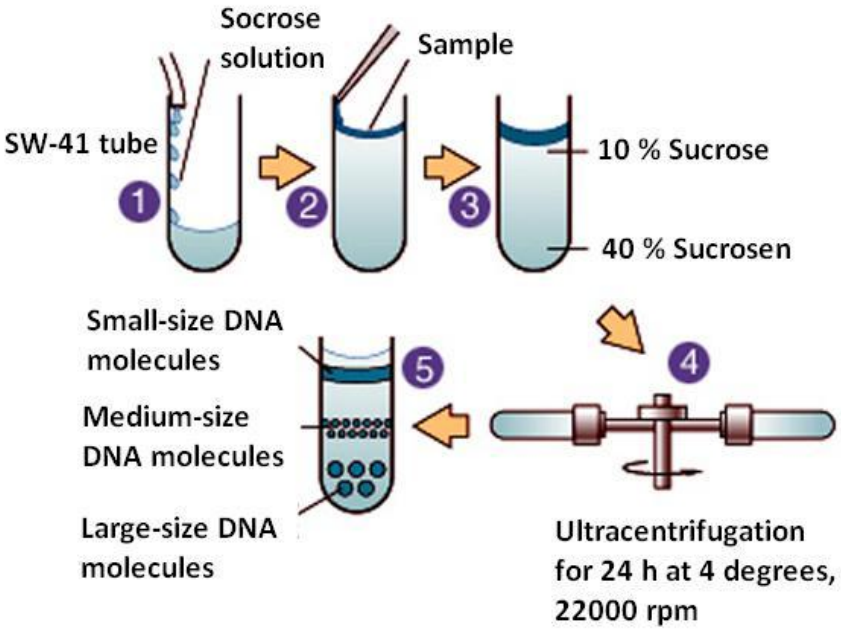

Figure 15: Steps in size fractionation by sucrose gradient (10-40%).

Using a peristaltic pump, the DNA fragments were collected in 600 µl fractions by pumping in 70% sucrose from the bottom. Pumping in 70% sucrose solution eventually pumped out the gradient, the heavier fragments came out first.

After collecting the fractions, samples of 40 µl of sucrose gradient were analyzed by 1 % agarose gel electrophoresis at (see Section 3.6.4).

3.6.3 Precipitation of DNA

Precipitation of DNA with ethanol is a standard method to concentrate the nucleic acids from aqueous solutions (Sambrook and Russell 2001). The fractions obtained from sucrose gradient were precipitated by adding 1 ml cold ethanol and 320 µl water to new tubes with ~180 µl of sucrose/DNA solution and placed at -20°C overnight. Next day, the tubes were centrifuged and pellets were resuspended with a total volume of ~100 µl TE buffer. Concentration of the DNA fragments was determined with NanoDrop 1000 (see Section 3.5.3) and the solution was used for ligation or stored at -20°C.

3.6.4 Agarose gel electrophoresis

Gel electrophoresis is a method used to separate DNA fragments by their size (bp) in agarose gel with electrical current (Sambrook and Russell 2001). DNA molecules contain phosphate groups which are negatively charged and therefore migrate toward the positive electrode. The rate of migration depends on the size and the conformation of the DNA. Larger fragments migrate slower than smaller fragments, allowing separation of different sizes of DNA fragments.

Plasmid DNA that was examined in this study existed in supercoiled and linear conformations. Linear and supercoiled DNA migrates differently. The supercoiled DNA is compact and migrates faster than the linear DNA. Under certain conditions, plasmid molecules can join together and form dimer (two plasmid molecules) or multimer (more than two plasmid molecules). These molecules would be larger than a monomer, and therefore migrate slower in the agarose gel.

3.6.4.1 Preparing of agarose gel

Agarose gel was prepared by adding 1 g agarose to 100 ml 1X TAE buffer and then melting the agarose in a microwave until the solution became clear. A comb was placed on the gel casting tray, and the melted agarose was poured into the tray and cooled until it was solid. The comb was removed and the gel was then placed in the electrophoresis chamber containing 1X TAE buffer.

3.6.4.2 Loading and running the gel

Samples of ~20-40 µl were analyzed for different assays, where each sample contained ~0,2-1,5 µg DNA, 1 µg 2-Log DNA Ladder and 10% loading buffer. The samples were carefully pipetted into separate wells in the gel. The power supply was turned on to 100 V. The samples

were run until the blue dye approached about half the length of the gel, which took approximately 1 h for small gels and 1,5 h for medium gels.

3.6.4.3 Staining the gel

Staining DNA in agarose gels was performed by using GelRed ™ 10,000X solution. For small gels 30 µl GelRed ™ solution was added to 100 ml dH_2O, and for medium gels 90 µl GelRed ™ solution was added to 300 ml dH_2O. The gel was stained for ~15-20 minutes. After staining, the gel was visualized with UV-light and a photo was taken by UV illuminator apparatus.

3.6.5 Ligation

Ligases catalyze the formation of phosphodiester bonds between the directly adjacent 3'-hydroxyl and 5'- phosphoryl termini of nucleic acid molecules. DNA ligase is most commonly used to ligate a fragment of DNA into a plasmid vector and is a fundamental technique in recombinant DNA work (Sambrook and Russell 2001).

Genomic DNA fragments (4-10 Kb) were ligated to 200 ng pUN121 with insert to vector ratio of 3:1. The amount of the insert was calculated by using Equation 1.

Equation 1: Calculation of 3:1 fragment ratio of insert: vector

$$\frac{\text{x ng vector} \times \text{bp insert}}{\text{x bp vector}} \times 3 = \text{x ng insert}$$

The reaction was ligated with 200 U T4 DNA ligase, and buffered with 1X T4 DNA ligase buffer (1/10 of the reaction volume). The reaction was incubated for 16 hours at 16^{o}C and the enzyme was inactivated by incubating for 25 minutes at 65^{o}C.

3.7 Transformation

Transformation is a process where DNA is transferred into a bacterium. DNA molecules would normally not pass through a bacterial cell membrane. In order to be able to take up the DNA, the bacteria must be made competent first,(i.e. be able to take up the DNA). Two types of competent cells are known in the laboratory: electrocompetent cells and chemically competent cells.

3.7.1 Preparation of competent cells

3.7.1.1 Electrocompetent E. coli

Making electrocompetent *E. coli* cells involves washing the cells with water, which makes the cell membrane permeable due to the osmotic effect.

Overnight culture (1,5 ml) was diluted 1:100 in 400 ml low-salt LB-medium and incubated at 37ºC or 42ºC. OD_{600} measurements were taken until OD_{600} reached 0,8. The culture was transferred into centrifuge tubes and centrifuged for 6 minutes at 4ºC and 8400 rpm. The cells were afterwards washed twice with cold sterile water (200-400 ml), resuspended and centrifuged for 6 minutes at 4ºC, 8400 rpm. Then 8 ml 10% cold sterile glycerol was added to the tubes and centrifuged for 6 minutes at 4^{o}C, 8400 rpm. The cells were resuspended in 1,2 ml 10 % cold sterile glycerol and divided in aliquots of 100 µl in eppendorf tubes. The cells were frozen with $N_{(l)}$ and stored at -80ºC.

3.7.1.2 Chemically competent E.coli

Competent cells can also be made by treating the cells with $CaCl_2$ that introduces pores in the cell membrane making the DNA able to diffuse through the cell membrane.

Overnight culture (1,5 ml) was diluted 1:250 in 25 ml LB and incubated at 37ºC or 42ºC until the culture reached an OD_{600} of 0,3. The cells were incubated on ice for 10 minutes before centrifugation for 10 minutes at 4ºC, 5000 rpm. The cells were resuspended in 1 ml cold sterile 0,1 M $CaCl_2$ and 15% glycerol. After incubation for ~30 minutes or longer (the longer the better) the cells were divided in aliquots of 200 µl, frozen with $N_{(l)}$ and stored at -80ºC.

3.7.2 Transformation into competent cells

3.7.2.1 Electrotransformation

Electrotransformation is a physical technique that is used to get DNA molecules into cells with thick cell walls, such as *E. coli*. Electroporation involves exposing the mixture of cells and DNA to a high voltage electrical pulse that makes the cell permeable and therefore able to take up the DNA (Sambrook and Russell 2001).

1-2 µl DNA (up to 100 ng) was added and mixed with 30-50 µl thawed (on ice) cells and incubated on ice for 1 minute. The Gene Pulser Appratus was set on: 25µF, 2.5 kV Pulse and 400Ω Controller. The mixture of cells and DNA was transferred into cold 0,2 cm Gene Pulser Cuvette and placed in the cuvette holder and pushed into the chamber between the electrodes. The electroporation process was conducted by pushing two red knobs simultaneously on the Gene Pulser apparatus until a beep sound was heard. After electroporation, the cuvette was removed from the chamber and 300-1000 µl LB/SOC medium was added. The transformation culture was then diluted if necessary and plated on agar plates supplemented with appropriate antibiotic.

3.7.2.2 Chemical transformation

1-5 µl of the ligation mixture (100 ng DNA) was transferred to 50-250 µl chemically competent cells and incubated on ice for 30 minutes followed by a 30 seconds of heat-shock at 42°C without shaking. Afterwards, cells were incubated on ice for 2 minutes before adding 250 µl pre-warmed LB/SOC medium and incubating in shaking incubator for 1 hour at 37^{o}C and 300 rpm. 25-100 µl of the culture was then plated on LB-plates with antibiotics.

4 RESULTS

4.1 Genetic screen

Genetic screen is a tool that has been used over the years for discovering new genes. In this study, genetic screen is used to discover genes that code for proteins that might interact with SeqA protein and promote its function (see Section 1.4).

Genomic libraries are used in genetic screen and are constructed by cloning segments of genomic DNA ligated into a vector. Once the genomic library is constructed, a suitable amount of the library is transferred into a strain that is chosen for the screen due to its properties. After incubation at appropriate temperature, the transformants isolated might contain the interesting gene (Sambrook and Russell 2001).

4.2 Construction of genomic library

A genomic library contains fragments that represent the whole genome of an organism. In this study the genome of *Escherichia coli* was used. In order to construct the genomic library, DNA from a wild type *E. coli* (MG1655) was isolated and digested with EcoRI. Fragments ranging from 4-10 Kb in size were isolated by sucrose gradient and ligated into a multicpoy vector. The ligation mixture was then transformed into chemically competent cells and incubated overnight. Figure 16 shows a stepwise scheme of genomic library construction.

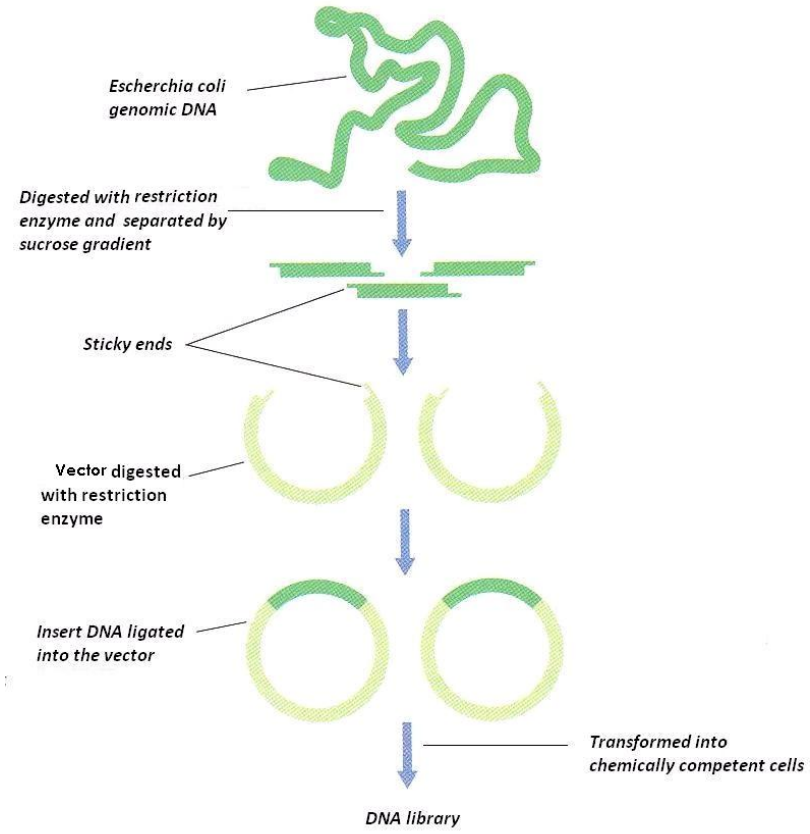

Figure 16: Steps in genomic library construction. The vector and insert were cut with the same restriction enzyme (EcoRI) and ligated together. This figure is modified from (Madigan, et al. 2008).

4.2.1 Selection and isolation of plasmid

An *E. coli* plasmid known as pUN121 was used as a vector in this study (Figure 17). pUN121 is a multicopy plasmid (15–20 copies per cell) with a size of 4,4 Kb, constructed by Nilsson et al. in 1983. pUN121 includes a tetracycline resistance gene under transcriptional control of a repressor protein coded by the phage *lambda cI* gene. In the absence of DNA inserted into pUN121, the repressor protein is expressed and binds to the promotor of the tetracycline resistance gene and prevents its transcription, causing the tetracycline gene to be switched off. However, when a DNA fragment is inserted, the repressor protein is not expressed, thereby allowing transcription of the tetracycline resistance gene. pUN121 also contains an ampicillin resistance gene.

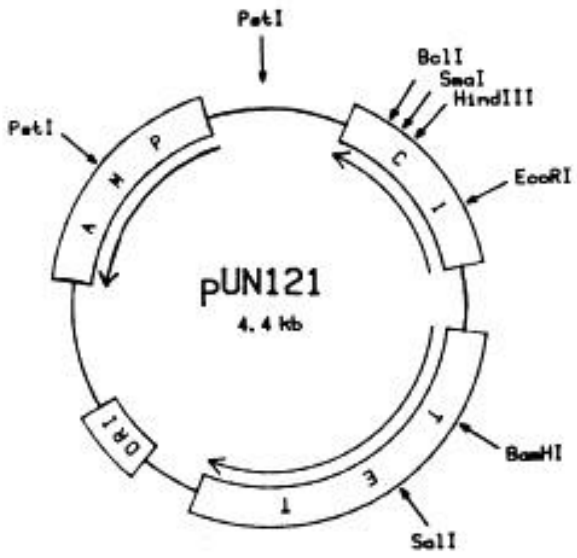

Figure 17: Plasmid map of *Escherichia coli* pUN121. "ORI" represents the origin of replication, "AMP" and "TET" represents ampicillin and tetracycline antibiotic resistance genes, respectively. "*cI*" represents the gene coding for repressor protein that regulates "TET" gene expression. pUN121 contains restriction enzyme cutting sites for such as EcoRI. This figure is from (Nilsson, et al. 1983).

pUN121 was isolated from ALO382 and purified with JETSTAR Plasmid Purification Midi Kit (see Section 3.5.1.1). 1 µg of purified pUN121 was digested with 10 U EcoRI and buffered with NEBuffer 4 (1/10 of the reaction volume). The reaction was incubated at 37°C for 2 hours, and inactivated by incubating at 65°C for 25 minutes (see Section 3.6.1). The final concentration of digested pUN121 was 50 ng/µl.

Agarose gel electrophoresis was performed to verify that pUN121 has been digested properly (see Section 3.6.4).. The gel was then analyzed under UV-light Figure 18 shows a fragment with a size of ~4,4 Kb in lane #2, which represents pUN121 that has been digested by EcoRI, and thus linearized. In lane #3, two fragments above 5 Kb were observed. These fragments belong to the undigested pUN121 and are suggested to represent the dimer- and multimer conformations of supercoiled pUN121. Due to its size, the multimer conformation migrates slower than the dimer conformation (see Section 3.6.4).

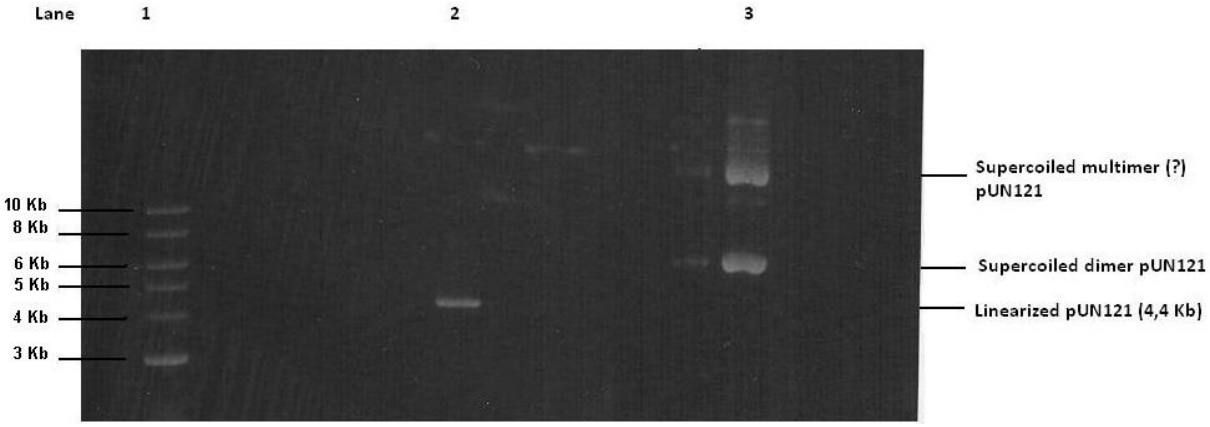

Figure 18: Gel analysis of pUN121. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 3 Kb to 10 Kb. Lane #2: pUN121(0,1 µg) from ALO382 digested with EcoRI. Lane #3: Undigested pUN121 (1,5 µg) from ALO382.

4.2.2 Dephosphorylation of pUN121

After digestion, pUN121 was dephosphorylation to prevent self ligation (see Section 3.5.4). The dephosphorylation reaction contained 1 µg EcoRI-cut pUN121, 5 U of antarctic phosphatase and 1X antarctic phosphatase buffer (1/10 of the reaction volume). The reaction was incubated for 15 minutes at 37^{o}C and inactivated by incubating for 5 minutes at 65^{o}C. The final concentration of dephosphorylated pUN121 was 33 ng/µl.

4.2.3 Isolation of DNA fragments

The complete genome of *E. coli* has been sequenced, and consists of 4,6 x 10^{6} bp (Blattner, et al. 1997). The genomic DNA used in this study was isolated from a wild type strain, MG1655. Genomic DNA was isolated with Wizard® Genomic DNA Purification Kit (see Section 3.5.2) and digested with EcoRI.

In order to obtain large DNA fragments, genomic DNA was cut with only one restriction enzyme. Larger DNA fragments might encode for several genes, and perhaps increase the

possibility of containing the gene of interest. However, since the gene and its location is yet unknown, there is a possibility that EcoRI cuts through the sequence of the unknown gene. Based on this speculation, it was suggested to perform the screen with different genomic libraries constructed by using insert fragments digested with different restriction enzymes. EcoRI was used in this study, whereas in another ongoing project, by Ida Benedikte Pedersen (personal communication), a library was constructed containing DNA fragments cut with HindIII. Consequently, the screen can be conducted usuing different type of DNA fragments.

After isolation of genomic DNA from MG1655, 200 µg DNA was cut with 10 U EcoRI and buffered with NEBuffer 4 (1/10 of the reaction volume). The reaction was then incubated at 37°C for 2 hours, and inactivated by incubating at 65°C for 25 minutes (see Section 3.6.1). Afterwards, an agarose gel electrophoresis analysis was performed to ensure that the DNA had been digested (Figure 19).

Figure 19 shows the digested genomic DNA in lane #2 with fragments <10 Kb and >1 Kb. The genomic library was constructed by including large DNA fragments ranging from 4-10 Kb in size (represented in a red box in Figure 19).

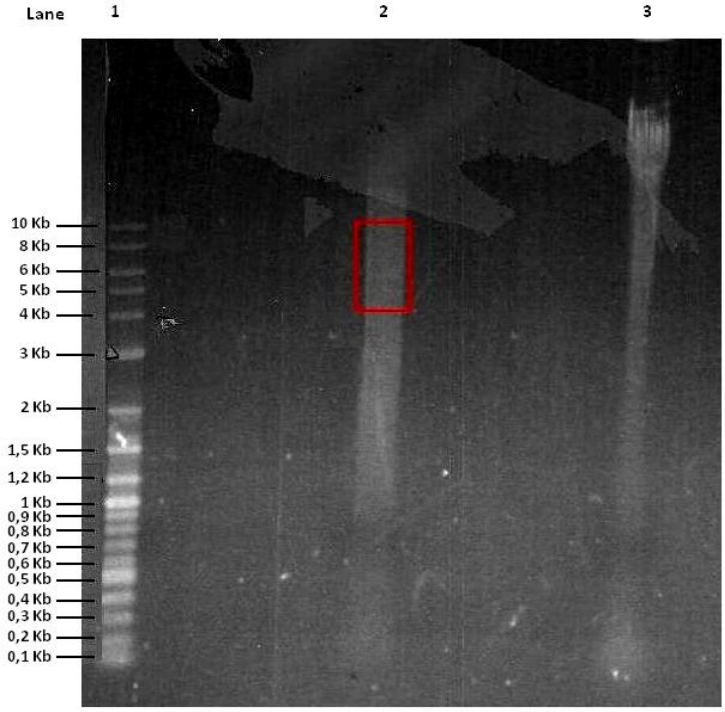

Figure 19: Gel analysis of digested and undigested genomic DNA fragments. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 100 bp to 10 Kb. Lane #2: Genomic DNA (10 µg) from MG1655 digested with EcoRI. Lane #3: Undigested genomic DNA (48 µg) from MG1655. The red box shows the fragments ranging from 4-10 Kb in size.

DNA fragments with size 4-10 Kb were therefore isolated by a 10-40% sucrose gradient (see Sections 3.6.2). 200 µg genomic DNA cut with EcoRI was applied to the 10-40 % sucrose gradient and centrifuged in an SW41 for 24 h at 4^{o}C, 22 000 rpm. The fragments were thereafter collected in 600 µl fractions (1-20). These fractions were analyzed with 1% agarose gel electrophoresis (see Section 3.6.4).

Agarose gel electrophoresis analysis showed that fractions 4-7 (Figure 19; lane #5-8) contained DNA fragments between 4-10 Kb. These tubes were combined and precipitated with ethanol (see Section 3.6.3). The concentration of the DNA fragments was measured using NanoDrop 1000 (see Section 3.5.3), and found to be 42 ng/µl. Fractions 12-20 were also analyzed, but no trace of DNA fragments was shown (Figure 21).

Figure 20: Gel analysis of DNA fragments after sucrose gradient. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 1,5 Kb to 10 Kb. Lane #2-12: 40 µl fraction number 1-11 from sucrose gradient. The red box represents the fragments of interest ranging from 4-10 Kb in size that were included in the genomic library.

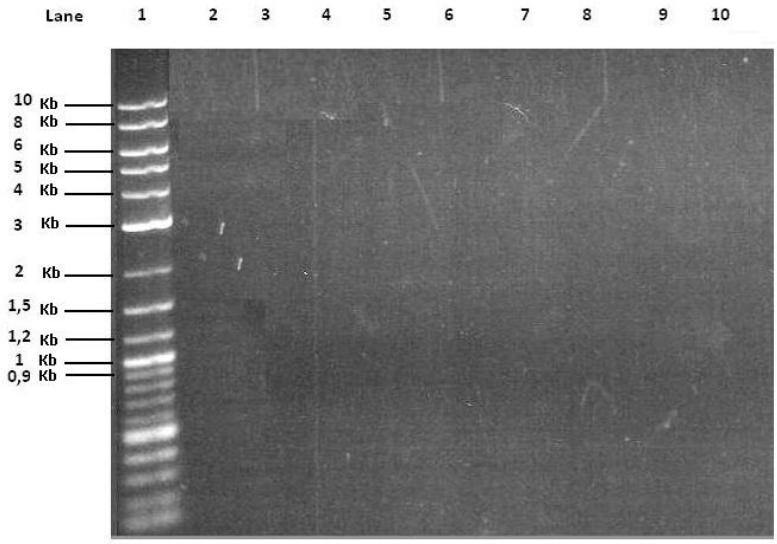

Figure 21: Gel analysis of DNA fragments after sucrose gradient. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 1,5 Kb to 10 Kb. Lane #2-10: 40 µl fraction number 12-20 from sucrose gradient.

4.2.4 Ligation of DNA fragments into pUN121

pUN121 (vector) and the appropriate genomic DNA fragments (insert) were digested with EcoRI, and thereby prepared for ligation. The next step was to find the correct amount of insert in order to obtain a 3:1 ratio of insert to vector. Equation 1 (see Section 3.6.5) was applied to calculate the correct amount of the insert to be ligated to 200 ng of cut and dephosphorylated pUN121. The average size of the inserts was 7 000 bp and used in Equation 1.

$$\frac{200\ \text{ng vector} \times 7\,000\ \text{bp insert}}{4\,400\ \text{bp vector}} \times 3 = 954\ \text{ng insert}$$

The ligation reaction therefore contained 954 ng of genomic DNA fragments (4-10 Kb) and 200 ng of digested and dephosphorylated pUN121 ligated by means of 200 U T4 DNA ligase and buffered with T4 DNA ligase buffer (see Section 3.6.5). The final concentration of the DNA in the ligation mixture was 28,8 ng/µl. Subsequently, the ligation mixture was transformed into cells with high transformation efficiency.

4.2.5 Transformation of ligation mixture

In order to obtain a successful transformation of a ligation mixture, it is necessary to use competent cells with high transformation efficiency. Two types of chemically competent cells were considered; One Shot® TOP10 and One Shot® OmniMAX™ 2 T1^{R}.

4.2.5.1 One Shot® TOP10 vs. One Shot® OmniMAX™ 2 T1^{R}

The transformation efficiency was tested by transforming 10 pg pUC19 into chemically competent cells (see Section 3.7.2.2). Different volumes of the transformation mixture were plated on LB plates with 100 µg/ml ampicillin and incubated overnight at 37°C. pUC19 has an ampicillin resistance gene and 100 µg/ml ampicillin was therefore used to select for transformants. Transformation efficiency was calculated by counting the number of colonies obtained the next day (Table 13). Equation 2 was used for this purpose. Table 13 shows the number of obtained transformants and calculated transformation efficiency.

Equation 2: Calculation of transformation efficiency

$$\frac{\text{Number of colonies}}{\text{pg transformed DNA}} \times 10^{6}\text{pg/µg} \times \frac{\text{Total transformation volume}}{\text{µl plated}}$$

$$= \text{Number of transformants per µg DNA}$$

Table 13 shows that One Shot® OmniMAX™ 2 T1^{R} had the highest transformation efficiency of 5,2 x 10^{8} transformants/µg DNA. One Shot® OmniMAX™ 2T1^{R} were therefore chosen for transformation of the ligation mixture.

Table 13: Transformation efficiency of One Shot® TOP10 cells vs. One Shot® OmniMax ™ 2 T1^{R} cells. The transformation efficiency was calculated by using the average number of colonies obtained from plating 1 µl, 10 µl and 100 µl of the transformation culture.

Chemically competent cells	Number of colonies			Transformation efficiency (number of transformants/µg DNA)
	1 µl plated	*10 µl plated*	*100 µl plated*	
One Shot® Top10	5	42	311	9,7 x 10^{7}
One Shot® OmniMax™ 2T1^{R}	121	394	1437	5,2 x 10^{8}

4.2.5.2 Transformation of the ligation mixture into One Shot® OmniMAX™ 2 T1^R

In order to obtain the genomic library, 100 ng ligation mixture was transformed into 50µl One Shot® OmniMAX™ 2 T1^R cells (Section 3.7.2.2). 750 µl LB medium was added before plating on 20 LB-agar plates with 5 µg/ml tetracycline (50 µl on each plate). The plates were incubated at 37°C overnight. As mentioned earlier, pUN121 has a tetracycline resistance gene that is repressed by *cI* repressor. When DNA is inserted into pUN121, *cI* repressor is not expressed the tetracycline resistance gene is transcribed (see Section 4.2.1). Tetracycline is therefore used to select for transformants of pUN121 with insert.

Growth was observed the following day, involving uncountable number of colonies that covered the entire surface of the plate. The colonies were scraped off the surface with a sterile glass spreader, transferred to 300 ml of LB with 5 µg/ml tetracycline and incubated with shaking at 37ºC overnight. The following day, samples from the overnight culture were stored as frozen glycerol stocks (750 µl overnight culture and 750 µl 87% glycerol) at -80ºC, and genomic library was isolated from the rest (~ 290 ml) with JETSTAR Plasmid Purification Midi Kit (see Section 3.5.1.1). The concentration of the genomic library was measured to be 841 ng/µl by using NanoDrop 1000 spectrophotometer (see Section 3.5.3).

4.3 Strain selection

After construction of the genomic library, a strain was selected for the screen, SF146. The SF146 strain contains two mutations, *seqA4* and *ΔrecA*. *seqA4* is a point mutation where alanine in the 25th position is replaced by threonine in the N-terminal domain (see Section 1.4.5), whereas *ΔrecA* is a deletion of the *recA* gene.

seqA deletion mutants have shown to have normal growth rate in poor medium, while in rich medium they grow slowly and form small colonies (Lu et al. 1994). However, this double mutant has shown to grow very poorly in a poor medium, and rich medium was therefore chosen instead.

Here, electrocompetent SF146 cells were prepared and OD_{600} measurements were taken at different time intervals until OD_{600} 0,8. Using data from OD_{600} measurements at exponential growth phase, a growth curve of SF146 was made (Figure 22).

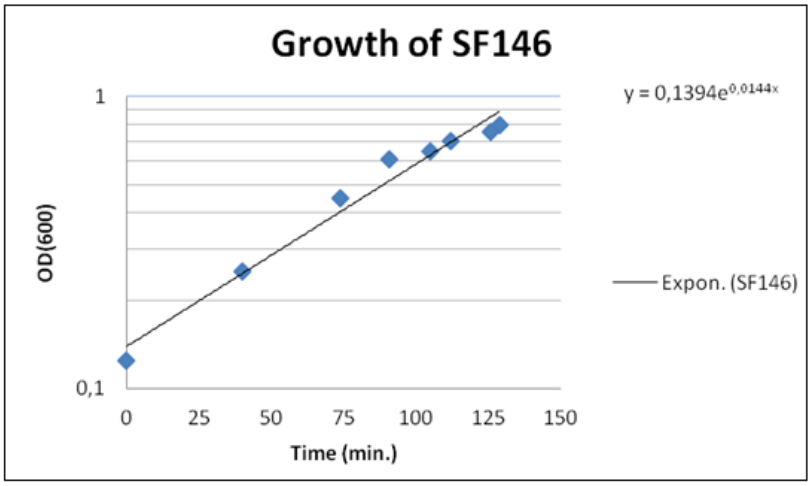

Figure 22: Bacterial growth curve of SF146.

We found that SF146 had a doubling time of about 50 minutes. This is slightly slower than the wild type *E. coli*. Wild type cells have a generation time of 20-25 minutes.

4.3.1 Viability testing of electrocompetent SF146 cells at different temperatures

SF146 cells are viable at 42°C, but not at 30°C (Solveig Fossum-Raunehaug, unpublished). The aim of this assay was to test the viability of this strain at 42°C and 30°C. Electrocompetent SF146 cells were used for this purpose.

10 µl of electrocompetent SF146 cells (not subjected to electroporation) were diluted (see Section 3.4) with dilution factors 10^{-1} to 10^{-4}. 100 µl from the last two dilutions were plated on LB agar plates with 5 µg/ml tetracycline and 30 µg/ml chloramphenicol (the SF146 strain has both tetracycline and chloramphenicol resistance genes). The plates were incubates at 42° and 30°C overnight for 2 days. Plates incubated at 42°C had an uncountable number of cells in all dilutions (>1000 colonies) from day 1, whereas plates incubated at 30°C were observed with a lower number of colonies. 31 colonies were obtained from the 10^{-4} dilution plate after one day of incubation, and 63 colonies after two days of incubation (Figure 23).

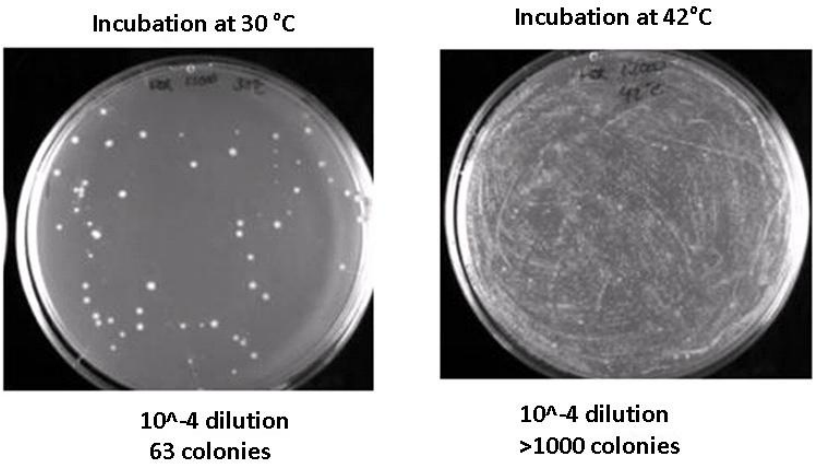

Figure 23: Growth of SF146 at 30°C and 42°C in the first viability assay. The pictures were taken after 2 days of incubation. Pictures from the first day had bad quality.

In order to obtain a countable colony number at 42°C, the assay was repeated using different dilutions (10^{-6} to 10^{-8}). Growth was observed after one day of incubation at 42°C (Figure 24 a), but not at 30°C. However, very small and clear colonies were observed after 2 days of incubation at 30°C. These colonies were marked and incubated for an additional night (to obtain bigger and countable colonies) and counted the next day. Incubation at 30°C after 2 days resulted in 47 colonies (Table 14), and after 3 days resulted in 63 colonies (Figure 24 b).

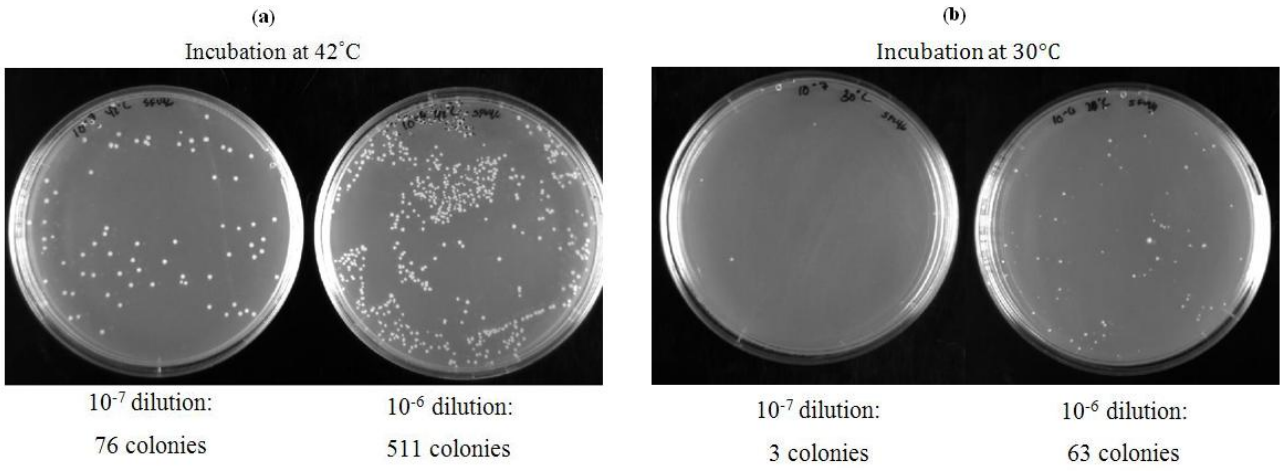

Figure 24: Growth of SF146 at (a) 42°C and (b) 30°C in the second viability assay. The pictures were taken after one day of incubation at 42°C, and after 3 days of incubation at 30°C. No pictures were taken after 1 and 2 days of incubation at 30°C.

Table 14 summarizes the number of c.f.u. after 1 and 2 days from the first and second viability assay at 30°C and 42°C in different dilutions.

Table 14: Number of c.f.u./plate form the viability assays at 30°C and 42°C. 100 µl from each dilution were plated on LB-agar plates containing tetracycline and chloramphenicol, 5 µg/ml and 30 µg/ml, respectively.

Dilution factor	Number of c.f.u./plate			
	Day 1 at 30°C	Day 1 at 42°C	Day 2 at 30°C	Day 2 at 42°C
First assay				
10^{-1}	>1000	>1000	>1000	>1000
10^{-2}	>1000	>1000	>1000	>1000
10^{-3}	231	>1000	314	>1000
10^{-4}	31	>1000	63	>1000
Second assay				
10^{-6}	0	511	47	571
10^{-7}	0	76	1	80
10^{-8}	0	1	0	3

An estimated number of cells plated was determined by OD_{600} measurements of electrocompetent SF146 (Table 15). The estimated number of cells plated and the number of c.f.u. per plate were used to calculate the frequency of c.f.u. (number of c.f.u. divided by the estimated number of cells plated).

The results from both assays showed that the SF146 strain is indeed viable at 42°C with frequency of c.f.u of 5,8 x 10^{-2} (obtained in the second assay), i.e. ~6 of 10^2 cells were able to form colonies. At 30°C, we found a frequency of 2,4 x 10^{-5} in the first assay (1 day of incubation), i.e. ~2 of 10^5 cells were able form colonies, and 3,6 x 10^{-3} in the second assay (2 days of incubation), i.e. ~4 of 10^3 cells were able to form colonies (Table 15).

Table 15: Number of SF146 cells/µl and frequency of c.f.u obtained from viability assays.

Assay	Estimated number of cells plated (measured with OD_{600})	c.f.u./plate	Frequency of c.f.u
At 30 °C, first assay	1,3 x 10^6	31*	2,4 x 10^{-5}
At 30 °C, second assay	1,3 x 10^4	47**	3,6 x 10^{-3}
At 42°C, second assay	1,3 x 10^3	76*	5,8 x 10^{-2}

* obtained after 1 day of incubation

** obtained after 2 days of incubation

4.4 Transformation efficiency of SF146

In order to have a successful screen, competent SF146 cells with high transformation efficiency are required. Both electro- and chemically competent cells were made in the lab and their transformation efficiency was determined using 10 pg pUC19. Electrocompetent cells showed higher transformation efficiency compared to chemically competent SF146 cells (data not shown). The next step involved determination of the optimal DNA concentration that yields the highest transformation efficiency in electrocompetent SF146. High transformation efficiency is important in order to increase the possibility of transforming the DNA fragment containing the unknown gene into SF146.

4.4.1 Transformation efficiency of electrocompetent SF146 cells with different DNA-concentrations

When DNA is transferred into competent cells, only a few cells in a population might take up the DNA. Therefore, it is very important to be able to transform SF146 with a high concentration of the genomic library. Different amounts between 10-100 ng were tested, and the DNA concentration that resulted in the highest transformation efficiency was used.

Since pUN121 was used to construct the genomic DNA library, it was decided to transform pUN121 with different amounts into SF146 in order to determine the transformation efficiency. 10 ng, 25 ng, 50 ng, 75 ng and 100 ng pUN121 were transformed into electrocompetent SF146 (see Section 3.7.2.1). Different volumes of the transformation cultures from each concentration were plated on LB-agar plates with 100 µg/ml ampicillin to select for transformants with pUN121 (see section 4.2.1). The plates were incubated at 42°C overnight, and the transformation efficiency was calculated based on the number of the colonies obtained the next day (Table 16). Equation 2 was used for this purpose (see Section 4.2.5.1).

Table 16: Transformation efficiency of SF146 with different amounts of DNA. Different volumes of the transformation reaction were plated with LB-medium to a total volume of 100 µl. Both positive control (electrocompetent SF146 on LB agar plates with 5 µg/ml tetracycline and 30 µg/ml chloramphenicol) and negative control (electrocompetent SF146 on plates with 100 µg/ml ampicillin) were included. ND: not determined. Colony number >1000 were counted by sectoring the plates and counting number of colonies in 1/6 or 1/8 of the plates and multiplying by 6 or 8.

Amount of DNA (ng)	Number of colonies				Transformation efficiency (number of transformants/µg DNA)
	0,5 µl plated	*1 µl plated*	*10 µl plated*	*100 µl plated*	
10	167	354	>1000	ND	$3,3 \times 10^7$
25	0	0	95	ND	$3,8 \times 10^5$
50	ND	~3296	Uncountable	Uncountable	$\sim 6,6 \times 10^7$
75	~2568	Uncountable	Uncountable	ND	$\sim 6,7 \times 10^7$
100	~1764	Uncountable	Uncountable	ND	$\sim 3,5 \times 10^7$

Based on the results from Table 16, a graph was plotted representing the transformation efficiency of SF146 with different amounts of DNA (Figure 25). Table 16 and Figure 25 show that 50 ng and 75 ng DNA yield the highest transformation efficiency of ~6,6 x 10^7 transformants/µg and ~6,7 x 10^7 transformants/µg, respectively. However, since it is desired to transform a large amount of the genomic library, 75 ng of the genomic library was therefore used for transformation.

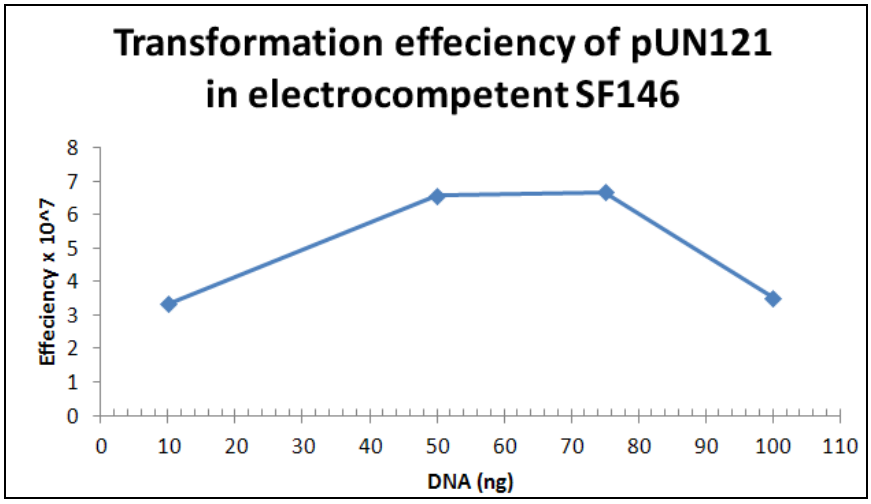

Figure 25: Transformation efficiency of SF146 with different amount of DNA.

4.5 Evaluation of the amount of genomic library

In order to have a successful screen, a high diversity of genes is required, i.e. high number of gene copies in the transformed genomic library. During transformation of the genomic library into the competent cells, only a few cells might take up the plasmid DNA that might contain the gene of interest. It is therefore important to have enough genes available to be taken up by the competent cells.

The aim here is to control if there are enough copies of the fragments in the genomic library that the SF146 cells might take up. The library contains 4,4 Kb vector and in average 7 Kb inserts; however, we are only interested in the amount of the insert fragments:

$$\frac{75 \text{ ng genomic library}}{11{,}4 \text{ Kb genomic library}} \times 7 \text{ Kb insert fragments} = 46 \text{ ng insert fragments}$$

The amount of the insert in the genomic library in bp is therefore:

1 pg = approx. 10^9 bp (Aarnes 2011)

46 ng = 46 000 pg= $4{,}6 \times 10^{13}$ bp insert fragments

The insert has a size between 4-10 Kb and must therefore calculate the number of the fragments with this size. Here we used the average size of the insert (7000 bp). Since 75 ng contain 4,6 x 10^{13} bp, the number of fragments with size of 7000 bp, that might contain the gene of interest, is therefore:

$$\frac{4{,}6 \text{ x } 10^{13} \text{bp}}{7000 \text{ bp}} = 6{,}5 \text{ x } 10^9 \text{ copies of insert fragments in 75 ng genomic library}$$

4.6 Transformation of SF146 with pUN121- background frequency

The viability of SF146 was tested at 30°C and 42°C, and the optimal transformation concentration was found. The next step was to transform pUN121 into SF146 and incubating at 30°C overnight. This test was important to get an indication of the growth frequency without any insert.

Before transformation of pUN121 into SF146, it was discovered that the plasmid pUN121 was isolated from ALO382 (*dam*$^-$), pUN121 was therefore transformed into a *dam*$^+$ strain (OmniMAX™ 2 T1^R), and isolated with a midiprep (see Section 3.5.1.1) for further use.

In order to be able to compare the results from this assay with the later results of the screen, the amount of pUN121 was calculated to be the same as the amount of the library (in base pairs) used for the screen. Equation 3 was applied to calculate the amount of pUN121.

Equation 3 Calculating of 1:1 pUN121 with insert: pUN121 without insert

$$\frac{x \text{ ng genomic library} \times \text{bp vector}}{x \text{ bp genomic library}} = x \text{ ng vector}$$

75 ng genomic library contains 11400 bp (in average 7000 bp insert and 4400 bp plasmid).

$$\frac{75 \text{ ng library} \times 4400 \text{ bp vector}}{11400 \text{ bp library}} = 28{,}95 \approx 29 \text{ ng vector}$$

29 ng pUN121 showed to be equal to 75 ng genomic library and was therefore transformed into 50 µl electrocompetent SF146 (see section 3.7.2.1). 1 ml transformation culture was plated on 10 LB- agar plates with 100 µg/ml ampicillin (100 µl on each plate) and incubated at 30°C overnight. No growth was observed after one day of incubation. Due to practical reasons, the plates were put in refrigerator for three days before putting them back for incubation at 30°C for an additional night. Growth was observed the next day. Table 17 shows the number of c.f.u./plate.

Table 17: Number of transformants from the background frequency (pUN121 transformed into SF146) colonies selected at 30°C. Obtained after 2 days of incubation.

Plate number	c.f.u./plate
1	230
2	200
3	62
4	17
5	>500
6	120
7	>500
8	>500
9	>500
10	70

The results showed an unexpected growth obtained by transforming only pUN121 in SF146. To ensure that these results were accurate, the test was repeated. No growth was observed after one day of incubation. However, after incubation for 2 days straight, growth was observed. Table 18 shows the number of c.f.u. on each plate.

Table 18: Second round of background frequency.

Plate number	c.f.u./plate
1	84
2	62
3	218
4	92
5	98
6	63
7	98
8	82
9	149
10	390

The results from Table 18 imply that the average number of c.f.u. is ~134/100 µl. Furthermore, the number of c.f.u. per plate varies between 62-390 colonies per plate. The results obtained from the first round were disregarded due to the incubation at 4°C for 3 days.

Plasmids (pUNK1 and pUNK2) from two colonies (K1 and K2) from the background frequency were analyzed by agarose gel electrophoresis (see Section 3.6.4). The plasmids were first purified with QIAprep miniprep (see Section 3.5.1.2) with concentrations measured to be 42,7 ng/µl and 40,9 ng/µl. pUNK1 and pUNK2 were then cut with EcoRI restriction enzyme in a 30 µl reaction (see Section 3.6.1) and analyzed with 1% agarose gel electrophoresis (Figure 26).

Figure 26 shows the linearized pUN121 in lane #2 with size of 4,4 Kb, and the supercoiled pUN121 in lane #3, as seen earlier, with a dimer- and multimer conformation (see Section 4.2.1). EcoRI-cut plasmids pUNK1 and pUNK2 in lane #4 and #6, respectively, showed to be smaller (< 3 Kb) than pUN121 (4,4 Kb). This observation was not expected, since only pUN121 was transferred into SF146, and pUNK1- and pUNK2 fragments were therefore expected to migrate with the same speed as linearized pUN121.

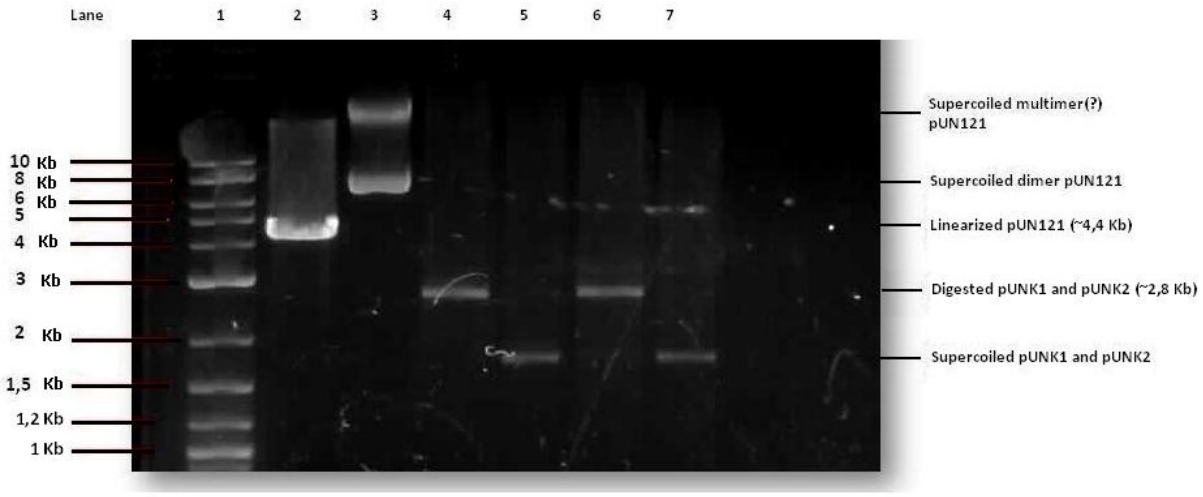

Figure 26: Gel analysis of pUNK1 and pUNK2 with pUN121 as a control. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 1 Kb to 10 Kb. Lane #2: pUN121 (1,7 µg) from OmniMAX™ 2 $T1^R$ digested with EcoRI. Lane #3: Undigested pUN121 (1,7 µg) from OmniMAX™ 2 T1R. Lane #4: pUNK1 (0,53 µg) from background frequency colony K1 digested with EcoRI. Lane #5: Undigested pUNK1(0,53 µg) from background frequency colony K1. Lane #6: pUNK2 (0,59 µg) from background frequency colony K2 digested with EcoRI. Lane #7: Undigested pUNK2 (0,59 µg) from background frequency colony K2.

4.7 Transformation of SF146 with genomic library- genetic screen

The DNA amount that resulted the highest transformation efficiency for electrocompetent SF146 cells has been determined to be 75 ng DNA with a transformation efficiency of 6,7 x 10^7 transformants/µg DNA (see Section 4.4.1). 75 ng of the genomic library was therefore transformed into electrocompetent SF146 cells. The transformation culture was then plated on LB-plates with 100 µg/ml ampicillin and incubated at 30°C overnight. The plates were checked for growth the following day and possibly incubated for an additional night.

The colonies subjected to further analysis were chosen due to size. More specifically, large colonies were picked out. The plasmids from these colonies were then purified and digested with EcoRI restriction enzyme, thus separating the insert from the plasmid. Gel electrophoresis was applied in order to analyze the insert that potentially could include a gene of interest (Figure 27).

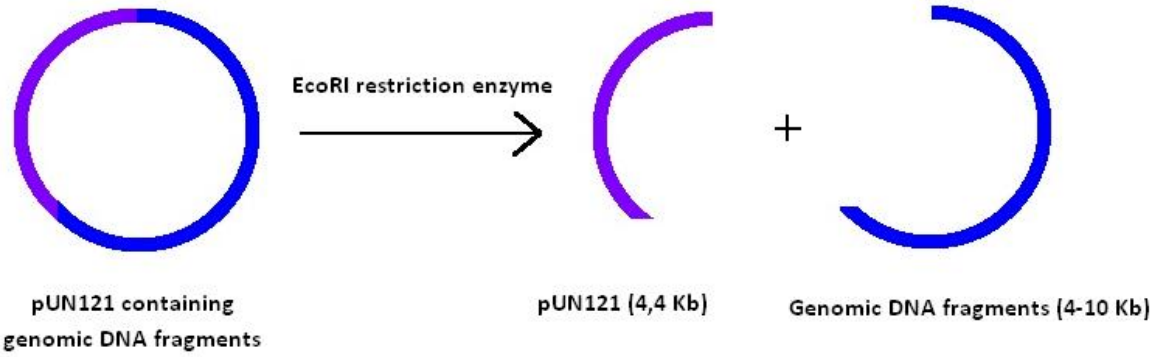

Figure 27: After selecting the transformants from the screen, the plasmids were purified and cut with EcoRI to separate the insert from pUN121.

Transformation of genomic library into SF146 was conducted 3 times to ensure that SF146 has been exposed to an adequate amount of genomic library. Based on our calculations (see Section 4.5). The fragment copy number was 6,5 x10^9. This indicates that the probability of transforming a gene of interest into SF146 is acceptable.

4.7.1 Genetic screen: first round

Here, 75 ng of the genomic library was transformed into 50 µl of electrocompetent SF146 cells. Then 1 ml LB was added and incubation continued at 42°C for 1,5 hour. The transformation reaction was then plated on 10 LB-agar plates with 100 µg/ml ampicillin (100 µl transformation reaction on each plate) and incubated at 30°C overnight. No growth was observed after one day of incubation, and the plates were therefore incubated for an additional night. The following day many colonies with different sizes were observed on each plate. Table 19 shows the number of colonies observed from the first screen.

Table 19: Number of transformants from first round of the screen.

Plate number	c.f.u./plate
1	338
2	338
3	601
4	305
5	465
6	321
7	692
8	397
9	370
10	335

The average number of c.f.u. from the first screen showed to be ~416 /100 µl with a variation of 305-692 colonies per plate.

Since it is very time consuming to analyze all colonies from the screen, it was decided to analyze only some colonies; the biggest colonies were chosen.

The plasmids from 20 screen colonies were purified and cut with EcoRI restriction enzyme (see Section 3.6.1). The cut plasmids were then loaded to a 1 % agarose gel and electrophoresis analysis was conducted (see Section 3.6.4).

Figure 28 shows the gel analysis of 20 colonies from plates 1-10 from the first round of the screen. The results showed the presence of a single band in colonies 1-20. All of the bands

had the same size of ~4,4 Kb. This band probably represents linearized pUN121, and no DNA fragment was observed.

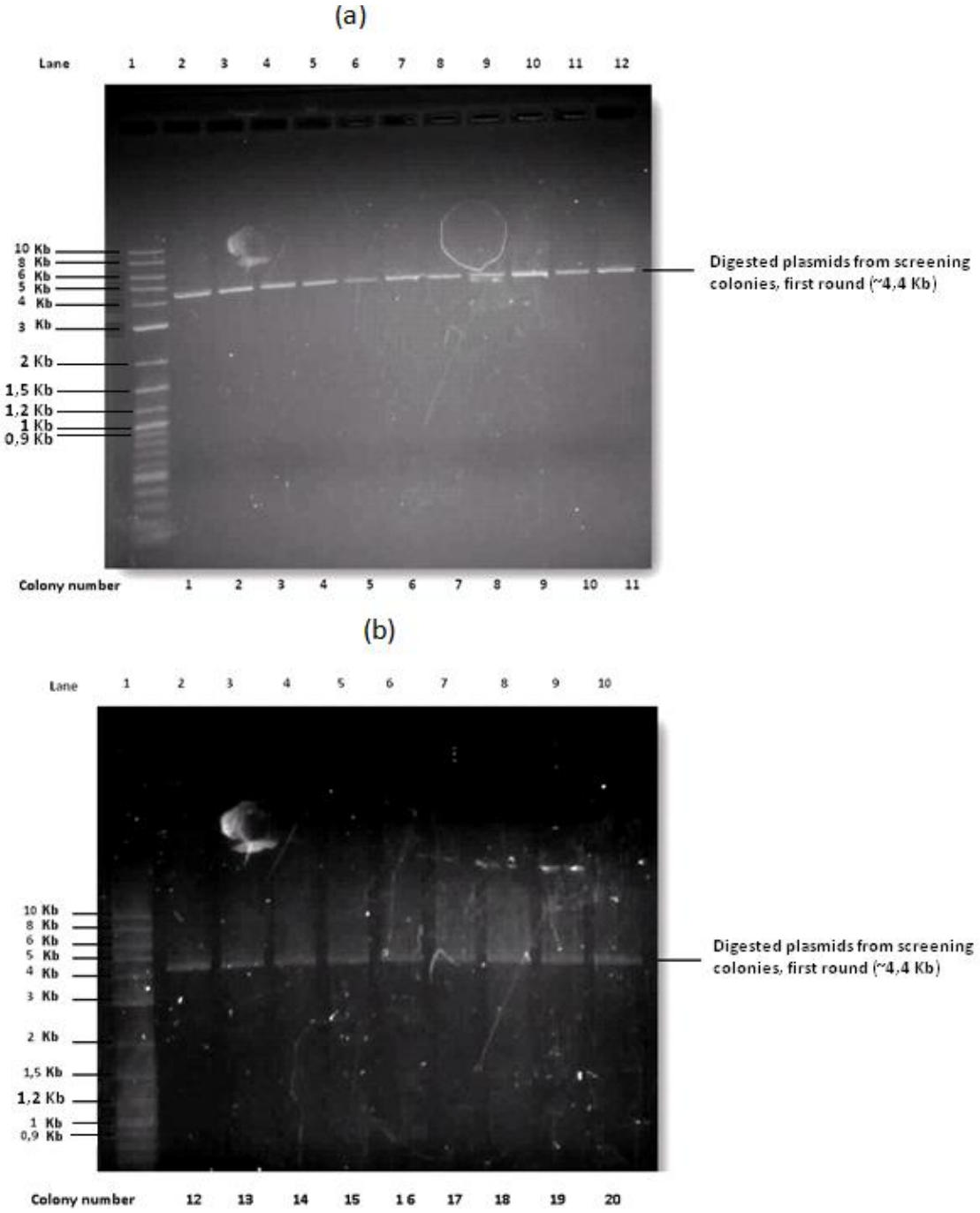

Figure 28: Gel analysis of 20 colonies from the first round of the screen. (a) Gel analysis of colonies 1-11 (from plates 1-6). Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 0,1 Kb to 10 Kb. Lane #2-12: Digested plasmid (0,37 µg – 0,77 µg) form colonies 1-11 **(b)** Gel analysis of colonies 12-20 (from plates 6-10) Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 0,9 Kb to 10 Kb. Lane #2-10: Digested plasmids (0,57 µg – 0,8 µg) from colonies 12-20.

We wanted to confirm that the colonies from the transformation of the genomic library contained pUN121. A new gel analysis was performed with two colonies from the first screen round, pUNKK1 and pUNKK2 (chosen from plate number 1), and pUN121 as a control (Figure 29).

Figure 29 shows that EcoRI-cut pUNKK1 and pUNKK2 have the same size as pUN121 (4,4 Kb). This observation indicates that bands in lane #4 and #6, are most likely digested pUN121 without insert. The gel analysis shows that the supercoiled conformations of pUNKK1 and pUNKK2 migrated differently (Figure 29; lane #5 and #7).

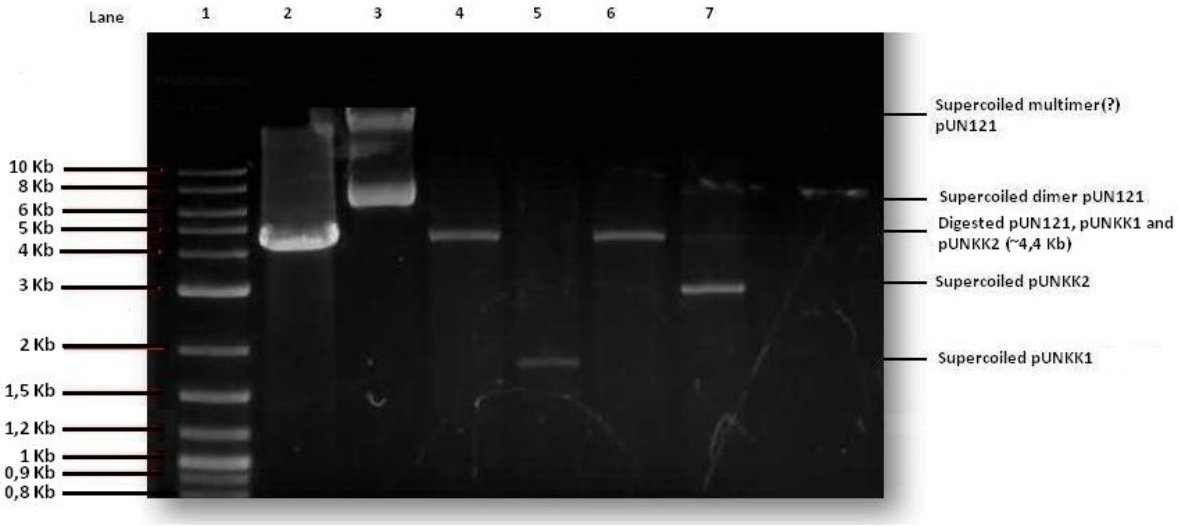

Figure 29: Gel analysis of pUNKK1 and pUNKK2 with pUN121 as a control. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 0,8 Kb to 10 Kb. Lane#2: pUN121 (1,7 µg) from OmniMAX™ 2 T1^{R} digested with EcoRI. Lane #3: Undigested pUN121 (1,7 µg) from OmniMAX™ 2 T1^{R}. Lane #4: pUNKK1 (0,5 µg) from screen colony KK1 digested with EcoRI. Lane #5: Undigested pUNKK1 (0,2 µg) from screen colony KK1. Lane #6: pUNKK2 (0,57 µg) from screen colony KK2 digested with EcoRI. Lane #7: Undigested pUNKK2 (0,45 µg) from screen colony KK2.

4.7.2 Genetic screen: second round

In the second round of screening, the same amount of genomic library was transformed into SF146, following similar procedures and conditions as in the first round (see Section 4.7.1). Similar to the results from the first screen, no growth was observed after one day of incubation and the plates were incubated for an additional night. Table 20 represents the results that were observed after two days of incubation.

Table 20: Number of transformants from the second round of the screen.

Plate number	c.f.u./plate
1	331
2	333
3	357
4	389
5	639
6	316
7	414
8	413
9	545
10	286

The average number of c.f.u. in the second round of the screen was ~402/100 µl. The number of the colonies per plate varied between 286-693.

Two colonies from the second round, KK3 (chosen form plate number 6) and KK4 (chosen form plate number 7), were exceptionally big compared to the rest of the colonies, and were therefore selected for analysis. Plasmids pUNKK3 and pUNKK4 were purified (see Section 3.5.1.2), cut with EcoRI (see Section3.6.1) and analyzed by agarose gel electrophoresis (see Section 3.6.4) (Figure 30).

Figure 30 shows that EcoRI-cut pUNKK3 and pUNKK4 migrates with the same speed as EcoRI-cut pUN121 (4,4 Kb), while the supercoiled conformations of pUNKK3 and pUNKK4, migrates faster than the dimer- and multimer conformations of pUN121.

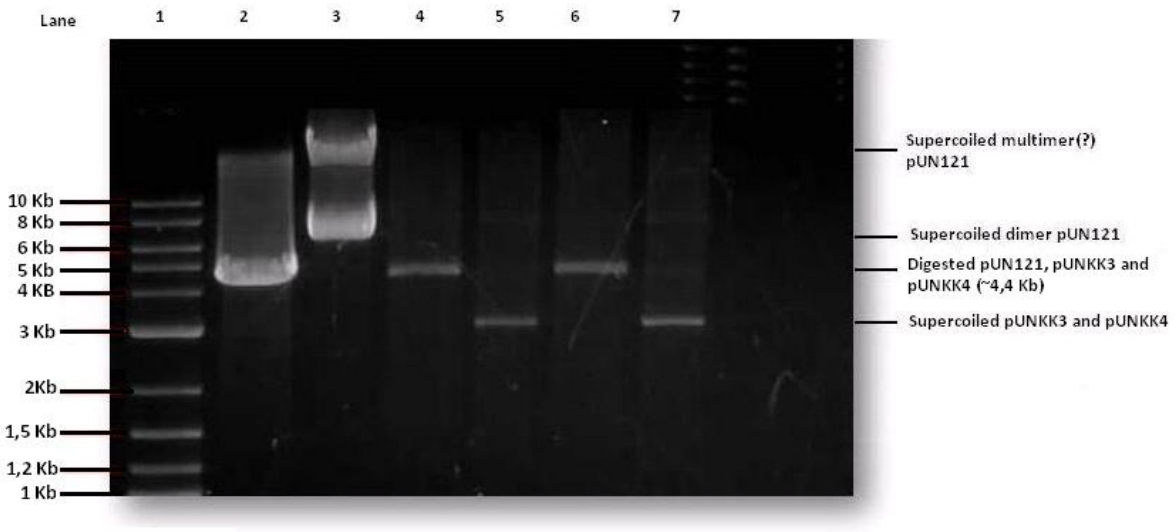

Figure 30: Gel analysis of pUNKK3 and pUNKK4 with pUN121 as a control. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 1 Kb to 10 Kb. Lane #2: pUN121 (1,7 µg) from OmniMAX™ 2 $T1^R$ digested with EcoRI. Lane #3: Undigested pUN121 (1,7 µg) from OmniMAX™ 2 $T1^R$. Lane #4: pUNKK3 (0,54 µg) from screen colony KK3 digested with EcoRI. Lane #5: Undigested pUNKK3 (0,54 µg) from screen colony KK3. Lane #6: pUNKK4 (0,54 µg) from screen colony KK4 digested with EcoRI. Lane #7: Undigested pUNKK2 (0,9 µg) from screen colony KK4.

4.7.3 Genetic screen: third round

Following the same steps of transformation as the first and the second round, a third and last round of screen was performed. Also there, the growth was observed after two days of incubation. Table 21 shows the results observed after two days of incubation with an average number of c.f.u. ~385/100 µl. The number of colonies per plate was ranging between 303-498 colonies.

Table 21: Number of transformants from the third round of screen

Plate number	Number of colonies
1	303
2	310
3	444
4	316
5	363
6	380
7	460
8	330
9	449
10	498

Three colonies were chosen from this screen, KK5 (from plate number 2) KK6 (from plate number 2) and KK7 (from plate number 4). Plasmids pUNKK5- pUNKK7 were purified (see Section 3.5.1.2), cut with EcoRI (see Section3.6.1) and analyzed with gel electrophoresis. (see Section 3.6.4) (Figure 31). Results from the agarose gel electrophoresis shows that EcoRI-cut pUNKK5, pUNKK6 and pUNKK7 had also the same size as EcoRI-cut pUN121.

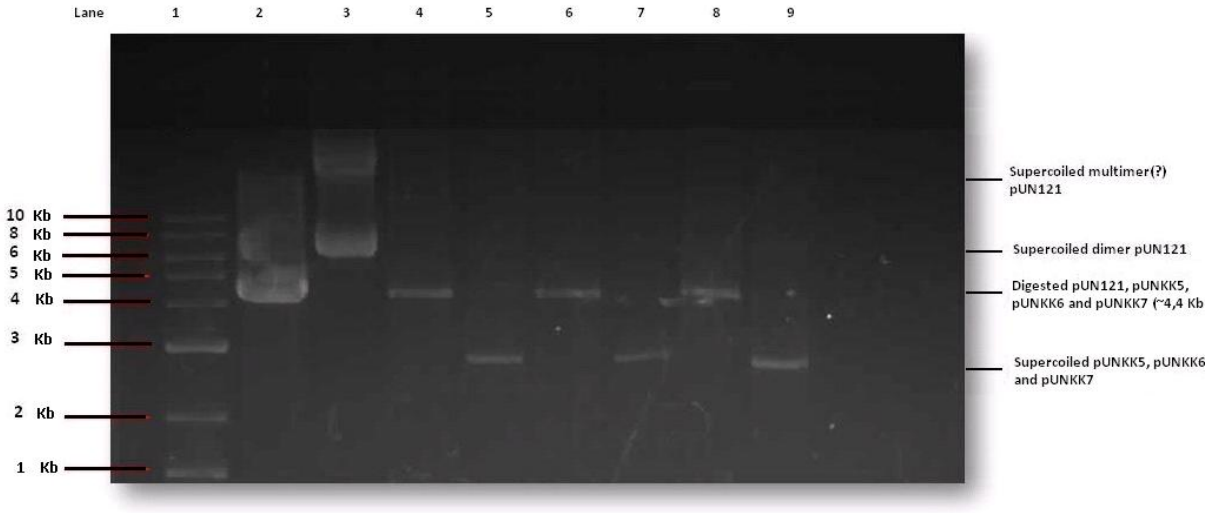

Figure 31: Gel analysis of pUNKK5, pUNKK6 and pUNKK7. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 1 Kb to 10 Kb. Lane#2: pUN121 (1,7 µg) from OmniMAX™ 2 $T1^R$ digested with EcoRI. Lane #3: Undigested pUN121 (1,7 µg) from OmniMAX™ 2 $T1^R$. Lane #4: pUNKK5 (0,7 µg) from screen colony KK5 digested with EcoRI. Lane #5: Undigested pUNKK5 (0,8 µg) from screen colony KK5. Lane #6: pUNKK6 (0,4 µg) from screen colony KK6 digested with EcoRI. Lane #7: Undigested pUNKK6 (0,4 µg) from screen colony KK6. Lane #8: pUNKK7 (0,5 µg) from screen colony KK7 digested with EcoRI. Lane #9: Undigested pUNKK7 (0,8 µg) from screen colony KK7.

In order to be able to compare the obtained results, Table 22 summarizes the estimated number of cells plated, c.f.u. obtained and frequency of c.f.u. at 30°C and 42°C form viability assays, background frequency and screening rounds. In viability assays, the electrocompetent cells were not subjected to electroporation prior to the plating on LB agar with 5 µg/ml tetracycline and 30 µg/ml chloramphenicol. The frequency of c.f.u. was $2{,}4x10^{-5}$ in the first round and $3{,}6 \times 10^{-3}$ in the second round.

Furthermore, the SF146 cells utilized in the background frequency assay and the screening rounds were added DNA, electroporated and plated on LB agar plates with 100 µg/ml ampicillin. The frequency of c.f.u. obtained in these assays were 2×10^{-7} and 6-6,4 $\times 10^{-7}$ for the background frequency and the screening rounds, respectively.

Table 22: Number of cells/µl and frequency of c.f.u. in different assays.

Assay	Estimated number of cells plated (measured with OD_{600})	c.f.u./plate at 30°C	Frequency of c.f.u.
Viability test of SF146, first round	$1{,}3 \times 10^{6}$	31*	$2{,}4 \times 10^{-5}$
Viability test of SF146, second round	$1{,}3 \times 10^{4}$	47**	$3{,}6 \times 10^{-3}$
Background frequency	$6{,}5 \times 10^{8}$	~ 134**▲	2×10^{-7}
First round of screen	$6{,}5 \times 10^{8}$	~ 416**▲	$6{,}4 \times 10^{-7}$
Second round of screen	$6{,}5 \times 10^{8}$	~ 402**▲	$6{,}2 \times 10^{-7}$
Third round of screen	$6{,}5 \times 10^{8}$	~ 385**▲	6×10^{-7}

*obtained after 1 day of incubation

** obtained after 2 days of incubation

▲ average number of c.f.u./plate is applied

4.7.4 Size-determination of pUNKK1-pUNKK7

Since no insert was observed in the agarose gel electrophoresis analysis of pUNKK1-pUNKK7, it was decided to run another agarose gel analysis to determine the size of the inserts. In order to determine the size of the plasmids, pUNKK1-pUNKK7 plasmids were cut with HindIII and therefore linearized *with* their inserts. HindIII has a restriction site in pUN121 (see Figure 17), and was therefore chosen for this purpose. Digestion with HindIII makes it possible to linearize pUNKK1-pUNKK7 without cutting out the insert. Figure 32 and 33 shows the results.

The gel electrophoresis analysis shows that linearized pUNKK1-pUNKK4 and pUNKK7 have approximately the same size as linearized pUN121 (4,4 Kb). However, pUNKK5 and pUNKK6 appeared to have a slightly bigger fragment (~ 4,5 Kb) compared to pUN121 (Figure 33).

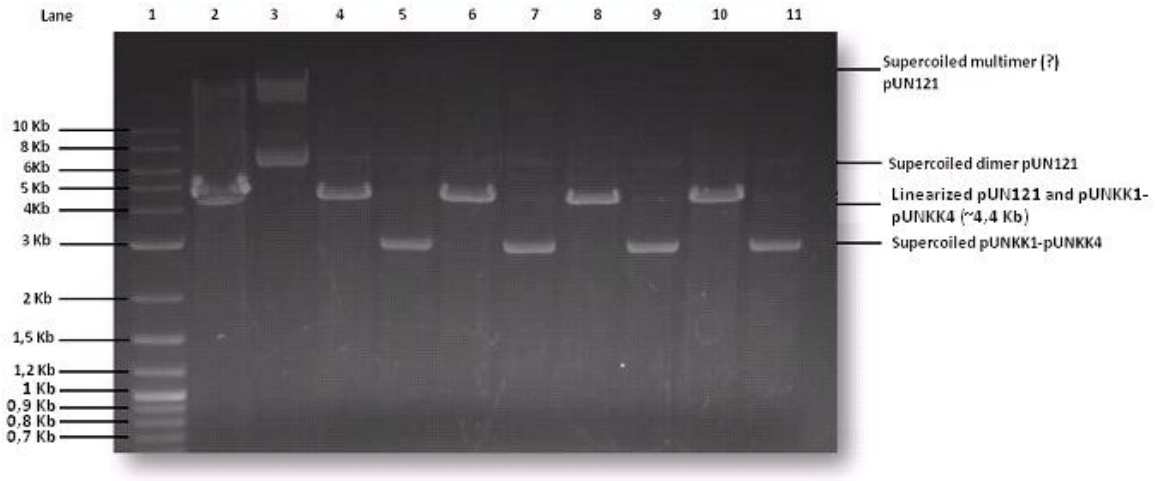

Figure 32: Gel analysis of pUNKK1-pUNKK4 cut with HindIII. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 0,7 Kb to 10 Kb. Lane #2: pUN121 (1 µg) from OmniMAX™ 2 T1^{R} digested with HindIII. Lane #3: Undigested pUN121 (1 µg) from OmniMAX™ 2 T1^{R}. Lane #4: pUNKK1 (0,9 µg) from screen colony KK1 digested with HindIII. Lane #5: Undigested pUNKK1 (0,9 µg) from screen colony KK1. Lane #6: pUNKK2 (0,9 µg) from screen colony KK2 digested with HindIII. Lane #7: Undigested pUNKK2 (0,9 µg) from screen colony KK2. Lane #8: pUNKK3 (0,8 µg) from screen colony KK3 digested with HindIII. Lane #9: Undigested pUNKK3 (0,8 µg) from screen colony KK3. #10: pUNKK4 (1 µg) from screen colony KK4 digested with HindIII. Lane #10: pUNKK3 (0,8 µg) from screen colony KK3 digested with HindIII. Lane #11: Undigested pUNKK4 (1 µg) from screen colony KK4.

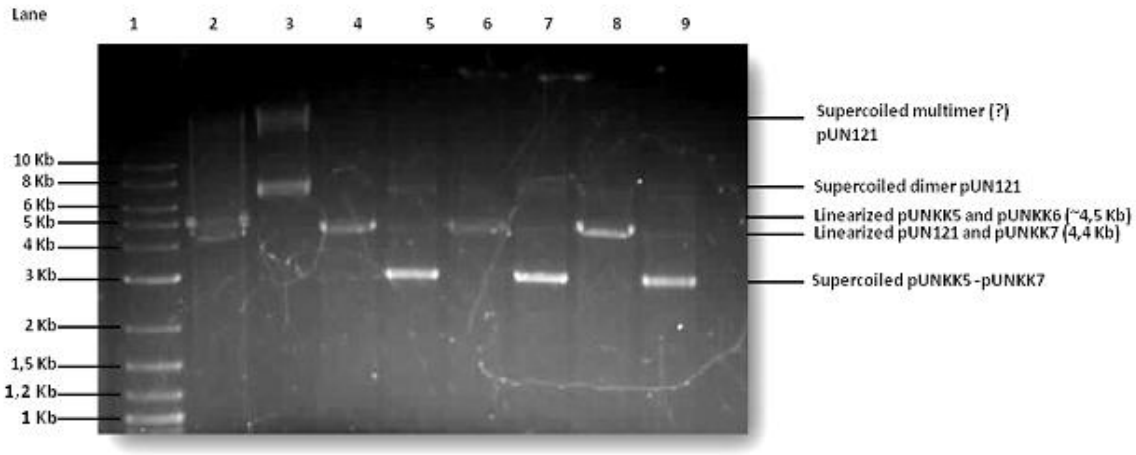

Figure 33: Gel analysis of pUNKK5-pUNKK7 cut with HindIII. Lane #1: 2-log DNA ladder (1 µg) with fragments ranging from 1 Kb to 10 Kb. Lane #2: pUN121 (1 µg) from OmniMAX™ 2 $T1^R$ digested with HindIII. Lane #3: Undigested pUN121 (1 µg) from OmniMAX™ 2 $T1^R$. Lane #4: pUNKK5 (0,7 µg) from screen colony KK5 digested with HindIII. Lane #5: Undigested pUNKK5 (0,7 µg) from screen colony KK5. Lane #6: pUNKK6 (0,8 µg) from screen colony KK6 digested with HindIII. Lane #7: Undigested pUNKK6 (0,8 µg) from screen colony KK6. Lane #8: pUNKK7 (0,9 µg) from screen colony KK7 digested with HindIII. Lane #9: Undigested pUNKK7 (0,9 µg) from screen colony KK7. The samples were run half the length of the agarose gel.

5 DISCUSSION

The aim of this study was to search for interacting partners for the SeqA protein. So far, SeqA is discovered to have an important role in sequestration by preventing reinitiation of replication of the newly synthesized origins (see Section 1.4.3.1). SeqA has also been suggested to have other roles, such as organization of the newly synthesized DNA and affecting the DNA topology (see Section 1.4).

It has been suggested that the SeqA protein does not operate alone. It would therefore be quite interesting to discover what type of proteins might help promoting the function of SeqA. In order to be able to search for such proteins, an *E. coli* genomic library was screened. First, a genomic library was constructed by ligating *E. coli* genomic DNA fragments ranging in size from 4-10 Kb into a multicopy plasmid. Large fragments between 4-10 Kb were used to increase the possibility of including the gene of interest in our genomic library. In order to select the transformants containing the gene of interest, a temperature-sensitive strain was used. The strain was viable at a specific temperature and died at another. The strain, SF146, has a *seqA* mutation and a *recA* deletion; *seqA4* Δ*recA*. The SeqA protein in the SF146 strain is still expressed, only with a dysfunction. However, the proteins we are searching for might be able to bind to the dysfunctional SeqA protein and promote its function.

The optimal growth temperature of a wild type *E. coli* is known to be 37°C, while the optimal growth temperature of SF146 strain is 42°C compared to 37°C (data not shown) and 30°C. The SF146 strain has a deletion of the *recA* gene, consequently lacking expression of the RecA protein. The RecA protein is important in several biological processes, such as DNA repair, homologous recombination and induction of the SOS response (see Section 1.5). It is suggested that the SF146 strain is viable at 42°C, although it lacks the *recA* gene, due to the expression of a number of proteins that help counteract the damaged DNA, termed heat shock proteins (Madigan, et al. 2008). The synthesis of such proteins is induced by heat stress in cells (Madigan, et al. 2008). Single mutants of *seqA4* (von Freiesleben, et al. 1994) or Δ*recA* have shown to be viable at 30°C (Kouzminova, et al. 2004), however, a strain with double mutation of *seqA4*Δ*recA* have shown to be dead at 30°C (Solveig Fossum-Raunehaug, unpublished data).

The *seqA4* mutation in SF146 is shown to cause asynchronous initiation of DNA replication and overinitation of replication (Odsbu, et al. 2005), which may lead to cell death due to collapse of the replication forks. Since SF146 also lack the RecA protein, the strain dies at 30°C. Experiments have shown that the *seqA4* mutant is able to form dimers of the SeqA proteins, but not multimers. However, it was observed that the synchrony of DNA replication was restored when high levels of SeqA4 were produced. These findings indicate that sequestration does not necessarily require the formation of SeqA-multimers, as long as the local concentration of the SeqA proteins is high enough (Odsbu, et al. 2005). It also shown that the *seqA4* mutant does not form foci and lacks therefore the stabilizing structure of SeqA behind replication forks. It is suggested that the SF146 strain is not viable at 30°C, due to the dysfunctional SeqA4 protein and the lack of RecA protein. Furthermore, heat shock proteins are not induced at 30°C.

The idea of the screen was to transform an adequate amount of the genomic library, containing large fragments that might include several genes, into the SF146 strain and selecting for transformants at 30°C. If the SF146 strain survived at 30°C, it would indicate that the plasmid contains the gene of interest coding for a SeqA-interacting protein.

5.1 Viability of SF146

In order to verify that the SF146 was viable at 42°C and not at 30°C, viability assays were conducted. The results showed that SF146 strain was indeed viable at 42°C, but also at 30°C only with lower frequency (Table 15). The frequency of c.f.u. at 42°C was 5,8 x 10^{-2}. At 30°C different results were obtained. The frequency of c.f.u. was 2,4 x 10^{-5} and 3,6 x 10^{-3} in the first and second assay, respectively. In addition, growth was observed after 1 day of incubation at 42°C in both assays. Whereas at 30°C, growth was observed after one day of incubation in the first assay, and after 2 days of incubation in the second assay. These results indicate that the SF146 strain is viable at 42°C, however at 30°C, different results could be obtained in different attempts.

5.2 Background frequency colonies

We wanted to investigate the frequency of growth when SF146 cells were transformed with pUN121 (empty vector). Basically, SF146 should not be viable at 30°C when an empty vector is transferred, thus growth was not expected in this assay. The results showed that no growth was obtained after one day of incubation at 30°C. However, after two days of incubation, SF146 were able to form colonies (Table 17).

The number of c.f.u. obtained from transforming pUN121 into the electrocompetent SF146 cells, varied between 17- >500 colonies per plate (Table 17). It was suggested that these results were inaccurate because of the way the plates were handled and the assay was therefore repeated. Furthermore, in the second round of background frequency assay, the number of c.f.u. varied between 62-360 colonies per plate (Table 18). Although a lower number of colonies were obtained the second time, the results were still not as expected. How can pUN121 alone promote the growth of SF146 at 30°C? It is suggested that perhaps one or several suppressors were involved. However, the question now is whether the suppressors present in the first assay are the same as in the second one? And also, in which step of the transformation process were the suppressors present? It is impossible to know *when* the suppression arose, whether the suppressors were present in the overnight culture, during preparation of the electrocompetent cells or during transformation.

Two plasmids, pUNK1 and pUNK2, from background frequency colonies K1 and K2 were analyzed by agarose gel electrophoresis, where pUN121was included as a control. Figure 26 shows the results from the gel electrophoresis, where in lane #2 the EcoRI-treated pUN121, thus linearized, had a fragment size of 4,4 Kb (see Section 4.2.1). Supercoiled conformations usually migrate faster than linear conformations, however, it was observed that the supercoiled conformations of pUN121 migrated slower than the linear conformation. It is therefore suggested that the bands in lane #3 represent the dimer and multimer conformations of pUN121. The dimer and multimer conformations of pUN121 were seen in all gels that included undigested pUN121 (Figures 18, 26 and 29-33; lane #3). In order to obtain a monomer of pUN121, the plasmid could be cut with a restriction enzyme followed by relegation and transformation into competent cells.

It was expected that EcoRI-cut pUNK1 and pUNK2 plasmids would have the same fragment size as pUN121 (4,4 Kb). Figure 26 shows that the EcoRI-cut pUNK1 and pUNK2 plasmids migrated to slightly below 3 Kb. These results indicate that the colonies obtained from the background frequency were possibly connected to the suppressor involved. The suppressor was somehow able to remove the unnecessary parts of the plasmid and only retaining the important parts. The important parts are probably the ampicillin resistance gene and the plasmid origin. In order to confirm the last assumption, sequencing of pUNK1 and pUNK2 is required.

It is suggested that since pUNK1 and pUNK2 were chosen from two different plates and yet behaved the same way, this might indicate that the suppressors were present in the transformation culture.

5.3 The isolated screen colonies

In a previous study, a bacterial two-hybrid system was used to search for SeqA-interacting proteins (Kang, et al. 2003). Two SeqA-interacting clones were identified, both expressed the C-terminal region of the ParC subunit of Topo IV. It was therefore possible that Topo IV would show up in our screen. In addition, biochemical studies have been conducted, where membrane fractions of a wild type *E. coli* and a Δ*seqA* mutant were isolated. Immunoprecipitation was then applied to search for proteins that would appear in samples from the wild type *E. coli*, but not in samples from the Δ*seqA* strain. Three proteins were

identified; aspartate transcarbamoylase and pyruvate dehydrogenase E1 and E2 (Solveig Fossum-Raunehaug, unpublished data). We were expecting to isolate one or more of those proteins in our genetic screen.

Genetic screening was conducted by transforming 75 ng of the genomic library into the SF146 strain. 75 ng genomic library contained 6,5 x 10^9 copies of the insert fragments, which was thought to be an adequate number of copies. The screen was conducted 3 times in this study, thus ~2 x 10^{10} copies of the insert fragment have been exposed to the SF146 strain.

Since the growth frequency of SF146 when transformed with an empty vector was investigated, we had a clearer picture of what would appear in the actual screen. We found that colonies were forming at 30°C after 2 days when transforming pUN121 into SF146. During genetic screening we looked for growth after one day of incubation at 30°C, which might indicate that the gene of interest was present. The genomic library was transformed into SF146, and growth selected at 30°C on LB-plates with ampicillin. It was expected that the cells containing the gene of interest would survive at 30°C. The results from the screening rounds showed that growth was obtained after 2 days of incubation at 30°C. The frequency of c.f.u. obtained from the screening rounds was 6-6,4 x 10^{-7}. The frequency of c.f.u. when empty pUN121 was transformed into SF146 (background frequency) was 2 x 10^{-7} (Table 22). This observation indicates that perhaps a number of these cells belong to the background frequency cells, while other cells might have the insert containing the gene of interest.

We found that the frequency of c.f.u. was lower in background frequency experiment and screening rounds compared to viability assays (Table 22). The cells from the background frequency and screening rounds have been subjected to electroporation in order to transform the DNA into the cells. Electroporation makes the cells weaker and it was therefore rational to obtain lower frequency of c.f.u. of cells subjected to electroporation, compared the cells from the viability test that were not subjected to electroporation.

Moreover, the cells from the viability assays were plated on LB agar plates containing tetracycline and chloramphenicol antibiotic, both of which the SF146 strain have resistance genes for. The cells from the background frequency and the screening rounds were plated on LB agar plates containing ampicillin, meaning that only the cells that had taken up pUN121 (with the gene of interest) would survive. Only a few cells in a population might take up pUN121 and be able to survive in ampicillin. It is therefore rational to obtain a lower

frequency of c.f.u. in the background frequency and screening rounds compared to the viability assays. However, since the growth was observed after 2 days of incubation in all assays, except in the first viability assay, it is suggested that a suppressor is involved in these assays. It is also suggested that perhaps different suppressors were involved in the first viability assay, compared to the second viability assay, background frequency and screening rounds.

Since >1000 colonies were obtained from each round of the screen, it was decided to select only a few colonies to analyze. The selection was according to size, where the largest colonies were selected. It was suggested that perhaps the largest colonies contain the gene of interest, making them grow with a normal size compared to the rest of the screen colonies.

Plasmids from 7 screening colonies (KK1-KK7), pUNKK1-pUNKK7, were isolated. The isolated plasmids basically consist of an insert ligated to a vector (pUN121). The plasmids were purified and treated with EcoRI so that the inserts were separated from the vector. Agarose gel electrophoresis was therefore expected to reveal two fragments from the digested screen plasmids, one representing the vector with a size of 4,4 Kb and the other one representing the insert we are interested in with a size of 4-10 Kb. Agarose gel electrophoresis of both digested and undigested pUNKK1-pUNKK7 and pUN121 (control) was conducted in order to investigate what type of insert each plasmid contained. The results showed that linearized pUN121 had a fragment size of 4,4 Kb, and as seen earlier, the supercoiled dimer- and multimer conformation that migrated slower than the linear conformation (Figures 18, 26 and 29-33; lane #3). The gel analysis also revealed that cut pUNKK1-pUNKK7 contained fragments of size 4,4 Kb. This represents the vector fragment. However, no other DNA fragments (insert) were observed (Figures 29-31). It was suggested that perhaps the inserts were too small to be seen on the gel.

In order to confirm whether an insert was present or not, another gel electrophoresis analysis was conducted, only this time the plasmids were digested with another restriction enzyme, HindIII. By treating the plasmids with another enzyme than EcoRI, the insert would still be attached to the linearized vector, thus obtaining larger fragment than 4,4 Kb. The results showed that the linearized pUNKK1-pUNKK4 and pUNKK7 had approximately the same size as linearized pUN121 (Figures 32 and 33). However, pUNKK5 and pUNKK7 had slightly bigger fragments than pUN121. These results indicate that small fragments were ligated into the pUN121 plasmids in pUNKK1-pUNKK7 instead of the larger fragments (4-10

Kb). However, it is difficult to determine the size of the inserts in pUNKK1-pUNKK7 based on the obtained results from the agarose gel (Figures 32 and 33).

The fragments were isolated by sucrose gradient, which involved separating different DNA fragments according to their sizes in a 10-40% sucrose gradient. After applying the genomic DNA (digested with EcoRI) into the gradient and ultracentrifigated, the DNA fragments were separated according to their size in different layers. The DNA fragments were collected in 600 µl fractions and analyzed with gel electrophoresis. Figure 20 shows the fragments collected from different fractions. Fractions 4-7 (Figure 20; lanes #5-8) contained fragments between 4-10 Kb and were therefore combined and used. However, in addition to fragments between 4-10 Kb, fractions 4-7 also contained smaller fragments, which had the possibility to be ligated into the vector.

At this point we knew that the screen colonies KK1-KK7 had taken up pUN121 with small insert fragments, which most likely do not code for something interesting. We also knew that the colonies survived without involvement of the gene of interest, and that one or several suppressors might have been involved in the background frequency. The results from the screen rounds might also indicate that one or several suppressor mutations were involved as well, due to the survival of the SF146 strain at 30°C without involvement of any gene of interest.

Supercoiled pUNKK1 was found to migrate very rapidly in the first gel analysis (Figure 29). However, in the second analysis of the plasmid, the supercoiled form migrated as expected (as supercoiled pUNKK2-pUNKK7) (Figure 32). Therefore, a suppressor mutation affecting supercoiling might be involved in the first round of analysis, but not present in the second round of growth for plasmid preparation.

In a genetic screening performed by Ida B. Pedersen (personal communication), a genomic library was transformed into three different tubes with electrocompetent SF146 using different amounts of the genomic library (1 µg, 1,5 µg and 2 µg). The transformation cultures were plated and incubated at 30°C. No growth was observed after one day of incubation. However, after 2 days of incubation it was observed growth in plates with 1,5 µg transformed genomic library, and no growth from the remaining plates (transformed with 1 µg and 2 µg genomic library). This observation might indicate that one or several suppressors might have been involved there as well. However, since growth was observed from only of the

transformations, the suppressor must have affected the cells under the transformation process and not during preparation of electrocompetent SF146 cells. The SF146 cells used in this study are assumed to have been subjected to suppressor mutations at an early stage.

5.4 Suppressor-problems with the SF146 strain

The SF146 strain was reported to be viable at 42°C and not at 30°C. However, in this study, we observed that electrocompetent SF146 cells were able to form colonies at 30°C (Table 15). As the results were unexpected, it makes us wonder what is it that makes the cells grow when they're supposed to be dead? Furthermore, unexpected growth was also observed from the later results in the background frequency, which made us think more about the selected strain.

The SF146 strain contains two mutations *seqA4* and Δ*recA*, where *seqA4* is a point mutation. It is suggested that one or several suppressors are involved, resulting in a new phenotype of the SF146 strain. Due to the changes made by the suppressor mutations at other locations in the genome than the original mutation, the new strain was shown to be viable at 30°C.

5.5 Further prospects

In order to have a successful genetic screen for SeqA partners, one must be able to avoid the suppressors that have shown to be a big problem in our screen. One way of obtaining that is by growing the SF146 cells in a minimal medium containing glucose with casamino acids (CAA) and the required amino acids (tryptophan, histidine, methionine). In a recent approach, by Ida B. Pedersen (personal communication), it has been shown that SF146 do grow in such medium with extremely slow growth rate compared to a rich medium such as LB.

Another prospect is that one could also perform the screen several times until growth is observed after one day of incubation. Nevertheless, there is also a possibility that the suppressors might also be involved.

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